The Dissertation Committee for Leah Renee Eller Certifies that this is the approved version of the following dissertation:

Anions in Hydrophobic Environments: Liquid-Liquid Extraction of Sulfate and Chloride, and Membrane Transport of Chloride

Committee:

Jonathan L. Sessler, Supervisor

Martin Poenie

Paul F. Barbara

Eric V. Anslyn

Nathan L. Bauld

Anions in Hydrophobic Environments: Liquid-Liquid Extraction of Sulfate and Chloride, and Membrane Transport of Chloride

by

Leah Renee Eller, B.S.

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Dedication

This dissertation is dedicated to my parents, Marie and Harry Eller, and my sisters, Rhonda, Chrystal and Suzannah, who have always supported me. I would not have made it this far without them.

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Wyeth, what can I say? I can't imagine graduate school without you. Thanks for the laughs and the fights. Good luck, my friend.

Thanks to all of the members of the Sessler group, past and present for the professional development, support and friendship over the years. Y'all are the best group in the world.

And, a special thanks goes out to the UT Judo Club. You gave me love and support over the years and a place to work out my stress. You taught me a lot more than just judo. You taught me about the person that I want to be. Stan, Ivy, Debbie, Chris Peter, Corey, Eric, Brandon, Brad, Dan and the rest of the gang. I love you guys and will miss you. Even you, Chris. ;)

Anions in Hydrophobic Environments: Liquid-Liquid Extraction of Sulfate and Chloride, and Membrane Transport of Chloride

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The transport of an anion across a lipid bilayer or the extraction of an anion into organic solution requires the stabilization of a charged species in a hydrophobic environment. Due to the similar energetic barriers of both processes, liquid-liquid extraction can potentially be used as a model for membrane transport. Carrier species that can efficiently extract anions from aqueous solutions into solvents such as chloroform, can potentially be utilized to facilitate the diffusion of those anions across a lipid bilayer or cell membrane. The research presented here explores the relationship between liquid-liquid extraction and membrane transport behavior. Chapter 1 presents an introduction to the equilibria reactions that are involved in extraction, the structure of lipid bilayers and a description of liposome models of cell membranes. Chapter 2 details the partitioning analysis of sulfate using radiotracers. Chapter 3 explores the chloride extraction behavior of several pyrrole-based molecules using radiotracer analysis. Chapter 4 details the extensive studies of chloride transport across lipid bilayers using liposome model membranes.

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Chapter 1: Introduction and Background

1.0 INTRODUCTION

The development of receptors for anionic species has proved to be particularly challenging for supramolecular chemists. Being comparatively large and charge-diffuse, anions are not bound as efficiently as cations by receptors.[1] While many researchers are now tackling the problem of anion recognition, through the introduction of pyrrole-based receptor systems, the Sessler group has made pioneering efforts in the area. Several of these systems have proven capable of effecting anion recognition in both nonpolar and polar media. Here, the bulk of the effort has focused on small, water-soluble anions such as fluoride, chloride, sulfate and phosphate. These anions are critical in a variety of biological processes and have been implicated in several medical ailments, such as fluorosis and cystic fibrosis; they are therefore viewed as important targets for the design of receptors and transport agents.[2]

One of the research goals within this area of supramolecular chemistry is to identify target compounds, which successfully bind anionic analytes of interest. Chloride,[3] fluoride,[4] phosphate,[5] and sulfate[6] have become popular targets for anion recognition chemists in the recent past because of their ubiquity in nature. Once appropriate receptor systems are identified, a second goal of this work involves correlating their structural features to their observed anion binding or transport functions. The determination of the specific design aspects that define the recognition behavior allows for improvements in function through the development of second and third generation receptors or carrier systems.

Supramolecular chemists approach recognition with various agendas. The goals of these chemists include the development of water-soluble recognition assemblies,[7-10] highly selective host species,[11-16] and sensors that allow for naked-eye detection[11,17-20] of the desired analyte, to name a few. Unlike much of the previous work in the Sessler Group, anion transport across a lipid bilayer is the primary focus of this dissertation. A variety of methods of study have been employed in this work to examine potential membrane transport agents. Liquid-liquid partitioning has been used as a model for transport events and to screen various potential target molecules. Transport has been directly studied using liposomes as a model for a living biological membrane. Several pyrrole-based molecules have been examined for both their anion extraction and anion transport ability. The purposes of the work presented in this dissertation are twofold: 1) to determine the structural aspects which effect the greatest anion transport and 2) to determine how strongly extraction behavior correlates with transport behavior (i.e. does liquid-liquid extraction offer a reasonable model of transport?)

The remainder of this chapter discusses the necessary background information relevant to the partitioning and transport studies that are presented in later chapters. A discussion of the utility of liquid-liquid partitioning as a model for transport events is also included. A fair amount of detail is given regarding the equilibria that govern the partitioning of ions into nonpolar phases, as well as the enthalpic and entropic considerations of ion solvation that are pertinent to this work.

1.1 BINDING FORCES

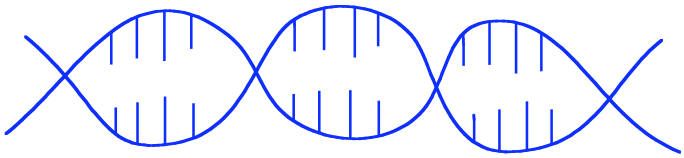
Nature, over the course of millions of years of evolution has developed very selective receptors for all manner of potential analytes. Nothing illustrates this perfection better than the enzyme-substrate interactions that have evolved over the ages. Here, a

combination of hydrogen bonding interactions, ionic attractions and van der Waals forces are employed, and it is generally through a combination of these binding modes that strength and selectivity in binding are achieved.[20]

1.1.1 Hydrogen Bonds

Hydrogen bonding arises from the sharing of a hydrogen atom between two electronegative atoms, generally F, Cl, O or N. This supramolecular interaction is charge-neutral, and can occur in both nonpolar and polar media. The greater the dipole of the solvent, the stronger a hydrogen-bond donor and acceptor must be in order to overcome competition arising from solvent molecules.

Hydrogen bonding has long been explored by the Sessler group as a molecular recognition tool. The development of sensors or transport agents based on hydrogen bonding interactions is both challenging and potentially rewarding. The challenge is to utilize neutral species to stabilize charged species. Ionic interactions are generally stronger, depending on the solvent environment, since opposite charges are inherently prone to associate. The development of a binding host that will effectively bind a cation or anion in the absence of oppositely charged moieties, requires careful consideration of geometry and the number of hydrogen-binding sites. A single hydrogen bond has a strength of approximately 1 kcal/mol in organic media.[21] To compensate for this weak interaction, Nature employs a large number of similar weak interactions in order to achieve overall strength in binding. A wonderful illustration of this practicality is seen in the DNA double-helix.



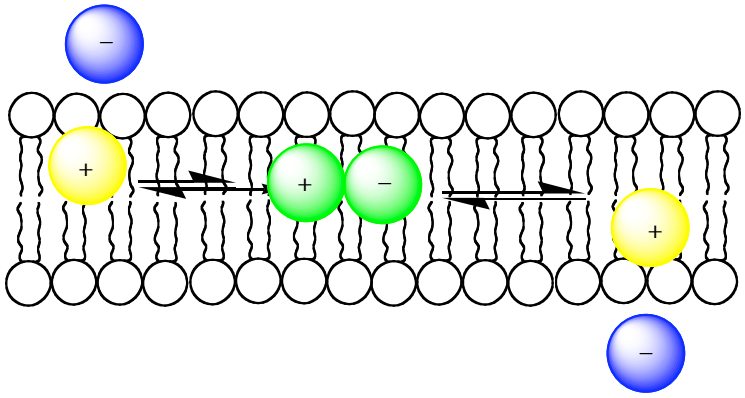
DNA is composed of a pair of organically-based strands with hydrogen bond donating and accepting sites. Through a combination of hydrophobic interactions, salt-bridges and hydrogen bonds, this complex is held together in water, a hostile solvent for hydrogen-bonded assemblies, with such strength that enzymatic catalysis is required to "unzip" the strands at biologically relevant temperatures.[20]

1.1.2 Ionic Attraction

Ionic attractions are often used by supramolecular chemists as recognition elements. Oppositely charged particles attract one another and generally interact in a noncovalent fashion. Ion-pairing is often the strongest of the noncovalent interactions. However, the strength of an ionic bond is highly dependent on the solvent in which the ion pair exists. The greater the dielectric constant of the solvent, the weaker the ion-pairing. Water is a hostile environment for ion-pairs, completely solvating each individual ion in multiple layers of solvent shells. With a dielectric constant of 78 Debye, most ion pairs will not form to any degree below a 1 M concentration in water. In nonpolar solvents with dielectric constants of approximately 30, typical ion pairs will begin to associate at 10^{-2} mol/dm^3. Inside a lipid bilayer, almost all ions exist as associated pairs.[22]

The exploitation of ion-pairing tendencies in various media is key to developing efficient anion extraction and/or transport processes. The utilization of cationic, lipophilic species to form strong contact pairs with the desired anion in nonpolar media,

while forming relatively weaker pairs in aqueous media, provides a convenient means of overcoming the energetic barriers associated with phase transfer.



Scheme 1.1 Ion-pairing interactions that may be exploited for use in through-membrane transport. The interior of a lipid bilayer is sufficiently hydrophobic that ion pairing is essentially 100%.

1.2 ENERGETICS OF ION SOLVATION

The issue of ion dehydration arises in both transport and extraction processes. The water molecules which form a solvent shell around the ion must be shed before that ion can easily enter a hydrophobic environment. For this reason, a brief discussion of the relevant energetics of such processes is pertinent at this juncture.

Generally speaking, an ion in aqueous solution is surrounded by several solvent shells of increasing disorder until the disorder of the shell equals that of the bulk solvent. The degree of charge-density, or hardness, of an ion dictates the number of shells and their corresponding level of order.

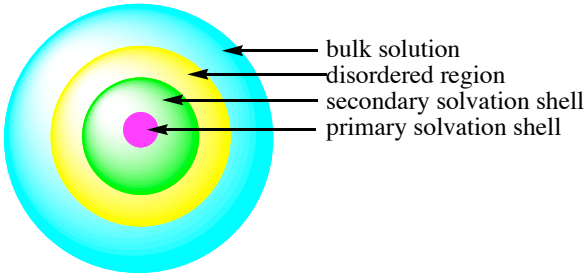


Figure 1.1 Solvation shell for an ion in aqueous solution. The degree of order decreases with distance from the ion until the disorder equals that of the bulk solution.

Harder ions are more strongly solvated and are known as structure-forming ions. That is, by aligning the dipoles strongly and packing the solvent molecules into a pseudo-crystal pattern, the overall entropy of the system is decreased. The desolvation of a structure-forming ion is entropically favored as the solvent molecules unpack and are returned to a random state. In water and other hydrogen-bonding solvents, entropy can have as much an impact on binding and transfer phenomena as enthalpy.[23] In the discussion that follows, energetic data for several cations and anions are presented. Our studies focus on chloride, which is presented along with ions of varying size, shape and charge density for the sake of comparison.

1.2.1 Partial molal hydration entropy

Standard partial molal entropies for hydrated ions provide a measure of the ability of an ion to break up or organize solvent molecules. Large, soft ions have large, positive partial molal entropies. These ions tend to break up structured solvents, such as water, and increase entropy. Small, hard ions have negative partial molal entropies. These ions

tend to be heavily hydrated and organize the solvent molecules into several levels of solvent shells, thereby decreasing the overall entropy of the system. The partial molal entropies for several cations and anions are summarized in Table 1.1.[24] Chloride has a partial molal entropy of +55 J/(K•mol). While certainly positive, the magnitude of the partial molal entropy is not overly large (over 100 J/(K•mol), meaning that the ability of chloride to affect solvent is relatively reduced. Chloride is somewhat structure-breaking. Therefore, transport of chloride out of aqueous solution is slightly unfavorable from an entropic perspective.

	J/(K•mol)		J/(K•mol)		J/(K•mol)
Li^+	+11	**Mg^{2+}**	-138	**Al^{3+}**	-322
Na^+	+59	**Ca^{2+}**	-53		
K^+	+101				
Rb^+	+120				
Cs^+	+133				
F^-	-10	**OH^-**	-11		
Cl^-	+55	**NO_3^-**	+125		
Br^-	+80	**SO_4^{2-}**	+17		
I^-	+109				

Table 1.1 Standard partial molal entropies (J/(K•mol)). Entropy data for hydrated ions in aqueous solution (relative to zero for the proton).

1.2.2 Partial molal hydration volume

Partial molal hydration volume is yet another measurable quantity which describes the solvation of an ion. Fundamentally, the partial molal hydration volume is a measure of the size of the solvent shell in which an ion exists in water. The size of the solvation shell of an ion is determined by two factors: ionic size and electrostriction. Electrostriction, as the name implies, results in a volume decrease for the solvent shell

due to the greater attraction solvent molecules have for charge-dense ions. Generally, small and medium-sized molecules with high charge have more negative partial molal hydration volumes that are dominated by electrostriction. Large, low-charge ions exhibit a solvation shell, the size of which is dominated by the size of the ion. As is shown in Table 1.2, chloride has a positive partial molal hydration volume, +17.8 cm^3/mol. Partial molal hydration volumes tend to correlate well with partial molal hydration entropies. As was the case with partial molal hydration entropy, relative to other ions, chloride is somewhat intermediate, being neither highly structured nor highly disordered in solution. Therefore, chloride can be viewed as a somewhat structure-breaking ion having a solvation shell, the diameter of which arises primarily from the size of the chloride ion, 1.81 Å. Various ionic radii are summarized in Table 1.3.[24]

	(cm^3/mol)		(cm^3/mol)		(cm^3/mol)
Li^+	-0.9	**Mg^{2+}**	-21.2	**Al^{3+}**	-42.2
Na^+	-1.2	**Ca^{2+}**	-17.9		
K^+	+9.0				
Rb^+	+14.1				
Cs^+	+21.3				
F^-	-1.1	**OH^-**	-4.0		
Cl^-	+17.8	**NO_3^-**	+29.0		
Br^-	+24.7	**SO_4^{2-}**	+14.0		
I^-	+36.2				

Table 1.2 Partial molal hydration volumes (cm^3/mol). Summary of the hydrated volume changes for various ions (relative to zero for the proton).

Cations	Å	Anions	Å
Na^+	1.02	F^-	1.33
K^+	1.38	Cl^-	1.81
Rb^+	1.49	Br^-	1.96
Cs^+	1.70	I^-	2.20

Table 1.3. Radii for various common ions.

1.2.3 Partial molal hydration enthalpy

It is entropically unfavorable for chloride to be removed from an aqueous environment and enter a hydrophobic environment. Entropy, however, is not the only energetic factor to be considered. The relevant enthalpic energies must also be examined.[24] As shown in Table 1.4, the hydration enthalpy for chloride is negative and therefore favorable, though not as favorable as the hydration enthalpies for other ions. While chloride is not as readily solvated as smaller, harder ions or ions of higher charge, hydration remains energetically favorable. Conversely, the dehydration necessary to transfer chloride to a hydrophobic environment would be energetically unfavorable.

Cations	kJ/mol	Anions	kJ/mol
Na^+	-405	Cl^-	-369
Li^+	-515	NO_3^-	-328
K^+	-321	SO_4^{2-}	-1145
Cs^+	-263		

Table 1.4 Hydration enthalpies for various ions.

1.2.4 Partial molal solvation enthalpy

The enthalpy of solvation in various solvents shows chloride to be fairly stabilized in polar, non-aqueous media, though not to the same extent as in water. This is highlighted by the data in Table 1.5, which shows a favorable, intermediate solvation in the case of the chloride anion. The lithium ion, being significantly harder, is solvated more readily; as is the barium ion. In fact, the barium(II) ion, being dicationic, is solvated more than four times as readily as chloride. This reflects the higher charge and is manifest, for instance, in the ability of this cation to interact more efficiently with solvents such as liquid ammonia.[24]

	Water (kJ/mol)	Methanol (kJ/mol)	Acetonitrile (kJ/mol)	DMSO (kJ/mol)
Li^+	-515	-531		
K^+	-321	-351	-347	-368
Cs^+	-263			
Cl^-	-369	-361	-349	-350

Table 1.5 Solvation enthalpies for various ions.

1.2.5 Partial molal transfer enthalpy

Given that chloride is less readily solvated in non-aqueous solvents, it follows that the enthalpy of transfer for chloride from water to a non-aqueous solvent is energetically unfavorable, i.e. it has a positive value. While the extent of this effect is small, it is certainly evident.[24]

The combination of the unfavorable decrease in entropy and the unfavorable increase in enthalpy that arises from removing a chloride ion from an aqueous environment and placing it into a non-aqueous environment clearly illustrates that a

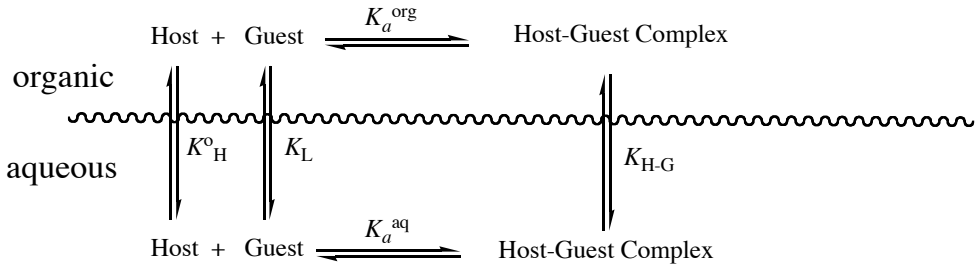
potential carrier or extraction agent with a respectable binding energy will be needed to overcome these energetic barriers and successfully displace the anion.

1.3 BINDING STUDIES

The ability of a molecule to bind an anion or other analyte can be quantified in a number of ways. Primarily, this is done by the determination of a binding constant, or association constant (K_a). Many analytical techniques can be employed to determine the binding affinity between two species. These techniques include, but are not limited to, NMR spectroscopy, UV-visible spectroscopy, and fluorescence spectroscopy. Due to the greater sensitivity of UV-visible and fluorescence techniques, and the high molar absorptivities and fluorescence efficiencies of the various anion receptor molecules developed in the Sessler group, the latter two techniques have been most frequently employed. Nonetheless, the experimental bases for these and the other measurement techniques mentioned above are basically the same. Briefly, some experimental parameter related to the concentration of one or more species is measured, with the changes in this signal upon addition of the anionic analyte to a solution of host being monitored. Typically, the solution that is titrated into the host also contains the host at the same concentration in order to keep the overall concentration of host constant throughout the titration, thus mitigating any potential dilution-related effects. The changes in the data signal are plotted against the concentration of analyte added in order to generate a binding isotherm. From these data, a best-fit curve can be derived and the binding constant, K_a, can be determined.

1.4 EXTRACTION STUDIES

Liquid-liquid partitioning offers another method of studying binding interactions. Partitioning is generally exploited by the organic chemist in synthetic workups, whereupon the desired organic molecule is dissolved in an organic solvent and washed with various aqueous solutions to remove salts and other impurities. The organic chemist relies on the favorable partitioning affinity of the desired molecule for the organic phase. The partitioning work in this dissertation relies on the same basic principle, but from the opposite viewpoint. The goal is to stabilize charged ions in organic solution using an appropriate extraction agent. A generic scheme of partitioning equilibria is shown below.



Scheme 1.2 Generic illustration of the relevant equilibria involved in liquid-liquid partitioning.

The efficiency of the extraction is dependent on the partitioning behavior of the guest species unassisted versus that of the host-guest complex.[25] This can be expressed by Eqn. 1.1,

$$k_{H\text{-}G} = k^o_H \left[\frac{(1 + k_a^{org}[G]^{org})^N}{(1 + k_a^{aq}[G]^{aq})^N} \right] \qquad (1.1)$$

where K^{o}_{H} = partition coefficient of the free host, K_a^{org} = binding constant in the organic phase, K_a^{aq} = binding constant in the aqueous phase, $[G]^{org}$ = concentration of guest in the organic phase, $[G]^{aq}$ = concentration of guest in the aqueous phase, and N = number of equivalent binding sites in the host. In the studies that are detailed in this dissertation, N = 1. This is common for synthetic systems. However, N is typically greater than one for protein-ligand interactions in which the protein has multiple binding sites.

Partitioning can be potentially useful as a model for transport processes. The mobilization of an ion from an aqueous environment into a hydrophobic environment can be viewed as an analogue of one-half of the transport process, specifically, moving the ion into the interior of a hydrophobic bilayer. The same energetic barriers apply in both cases. Thus, in this work the partitioning behavior of potential carrier molecules were first examined by liquid-liquid extraction. After screening the molecules in this manner, prospective candidates were then subjected to transport experiments using liposomes.

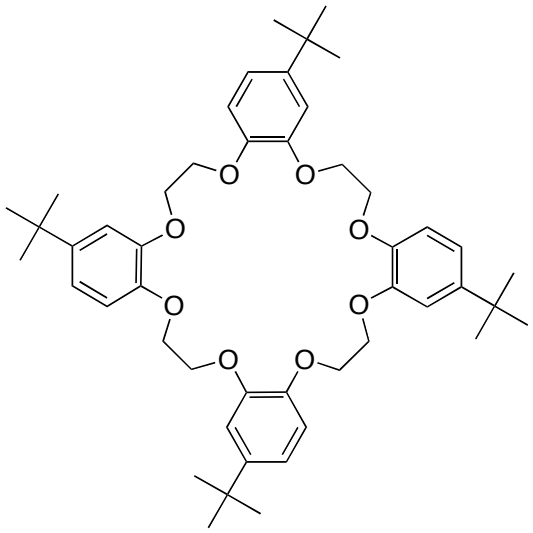
1.4.1 The Hofmeister effect

The physical chemistry data for ion solvation discussed in Section 1.2 can be summarized in terms of the Hofmeister effect.[26] Simply put, the Hofmeister effect describes an empirical phenomenon, namely that larger, softer anions, such as nitrate and perchlorate, are more readily extracted into organic media than smaller, harder anions such as chloride and fluoride. Intuitively, this effect makes good sense. Hard ions are more strongly solvated in water, having their charge more fully stabilized by the hydrogen-bonding solvent. Thus, they are not likely to be as well stabilized or solvated in a nonpolar environment. The Hofmeister series, that is, $ClO_4^- > NO_3^- > SO_4^{2-} > Br^- > Cl^- > F^-$, provides a reproducible, albeit qualitative reflection of the extractability of

various anions. Chloride, though softer than many cations, is still considered a hard anion by the extraction community. Favorable partitioning of chloride into organic solution remains a challenge. We believe that favorable partitioning into a hydrophobic environment, as is the case with liquid-liquid extraction, represents a first step towards favorable membrane transport because of the similar energetic barrier, i.e. moving a charged species from an aqueous to a non-aqueous environment.

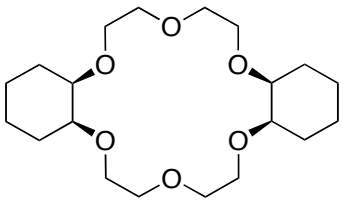
1.4.2 Previous works

The Chemical Separations Group at Oak Ridge National Laboratory under the supervision of Dr. Bruce A. Moyer has long been interested in effecting the removal of specific ions from aqueous solutions. They have, for instance, developed a variety of crown ether compounds capable of extracting cesium cations. Using compounds such as **1.1**, Moyer and coworkers obtained distribution ratios for Cs^+ (D_{Cs}) on the order of 0.1 for $CsClO_4$ and 0.01 for $CsNO_3$, where the D-value equals the ratio of the concentrations of Cs^+ in the organic and aqueous phases.[27] Currently a more elaborate calixarene crown ether system, capable of extracting Cs^+, is being developed for large-scale use within the Department of Energy mission. An equally impressive accomplishment, is the development of selective extraction processes for hydroxide ions in the presence of more lipophilic ions, overcoming the Hofmeister bias.

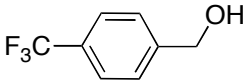


1.1

The hydroxide ion is particularly difficult to extract due to the high degree of solvation in the aqueous phase. The crown ether **1.2** is not capable of extracting hydroxide effectively ($D \approx 10^{-4}$) in accord with the Hofmeister bias. That is, Cl^-, Br^-, NO_3^- and ClO_4^- are extracted more readily than OH^-, with D-values between 10^{-3} to 10^{-2}. However, using fluorinated alcohols such as **1.3** in the organic phase, the Moyer group was able to overcome the Hofmeister bias and obtain higher distribution ratios for NaOH than any of the other sodium salts examined in the study.[28]

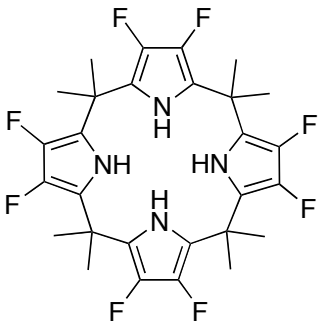


1.2

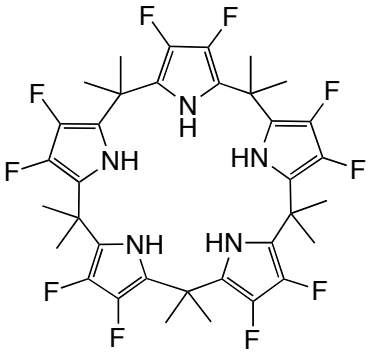


1.3

Using the fluorinated calixpyrroles, **1.4** and **1.5**, Moyer and coworkers were again able to overcome the Hofmeister bias. In this series of experiments, CsF was extracted into organic solution more readily than either CsCl or CsBr. The distribution ratios for CsF extraction were on the order of 10^{-3} or slightly larger, while the distribution ratios for CsBr extraction and CsCl extraction were both less than 10^{-3}.[29]



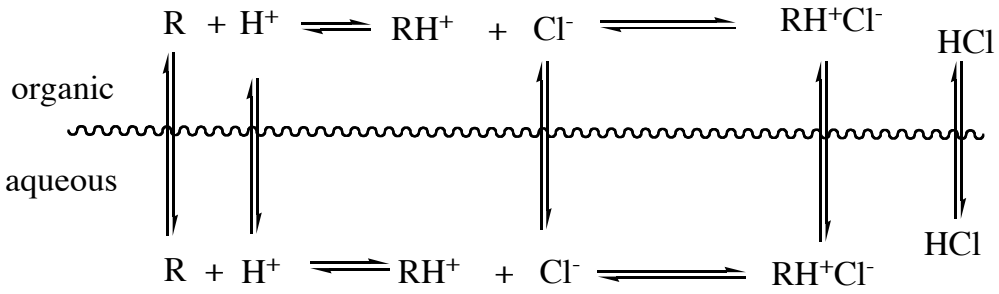
1.4



1.5

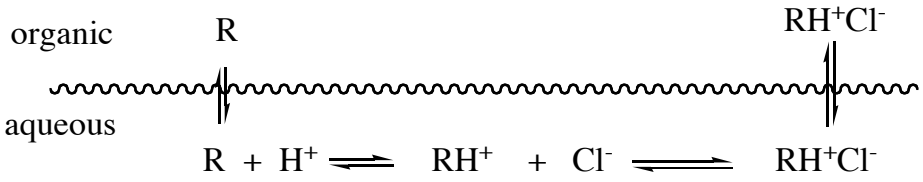
1.4.3 Chloride extraction equilibria

The work presented in this dissertation deals specifically with sulfate and chloride extraction, with a primary emphasis on extraction of the latter anion. Scheme 1.3 describes the relevant equilibria for chloride extraction using carriers that can protonate. In this scheme, a neutral extraction agent is protonated and the protonated species binds and extracts chloride into the organic layer. No density implication for the organic layer is intended in this generic scheme. Often chloroform was used and the organic layer was on the bottom.



Scheme 1.3 The relevant equilibria involved in chloride partitioning. The carrier molecule is represented by R and is shown in its free-base and protonated forms.

With a careful experimental design, the complex equilibria described above can be simplified. In particular, by using a highly hydrophobic extraction agent, the partitioning of the neutral carrier molecule can be rendered negligible. Additionally, using appropriate concentrations of salt, the amount of HCl relative to H^+ and Cl^- can be kept relatively low, thereby negating the partitioning of neutral HCl. Subject to such caveats, the equilibrium situation simplifies to that shown in Scheme 1.4,



Scheme 1.4 Simplified equilibria for chloride partitioning. This simplification is valid for nonpolar organic solvents and low-loading conditions.

where the equilibrium partitioning of R strongly favors the organic phase. The more polar the organic phase, the less valid such simplifications become in real life.

Derivation of the equilibrium equation for chloride extraction

If the distribution ratio of chloride, D, is defined to be the equilibrium concentration of chloride in the organic phase (a bar over a term indicates that the species it represents resides in the organic phase) divided by the equilibrium concentration of chloride in the aqueous phase,

$$D = \frac{[\overline{RHCl}]}{[Cl^-] + [RHCl]} \qquad (1.2)$$

and the complexation of extraction agent and chloride ion is

$$K_{cpx} = \frac{[RHCl]}{[RH^+]\,[Cl^-]} \; . \qquad (1.3)$$

The distribution ratio expression can be rewritten in terms of the aqueous binding constant as shown in Eqn. 1.4 where

$$D = \frac{[\overline{RHCl}]}{[Cl^-]\,(1 + K_{cpx}[RH^+])} \qquad (1.4)$$

the equilibrium ratio of the extraction of chloride is described by

$$K_{ex} = \frac{[\overline{RHCl}]}{[RH^+]\,[Cl^-]} = \frac{D_{Cl}\,(1 + K_{cpx}\,[RH^+])}{[RH^+]} \; . \qquad (1.5)$$

Rearrangement of Eqn. 1.5 gives

$$D_{Cl} = \frac{[RH^+]\, K_{ex}}{1 + K_{cpx}\,[RH^+]} \quad . \tag{1.6}$$

Taking the log of both sides and solving for k_{ex} gives

$$\log D_{Cl} = \log\left(\frac{[RH^+]}{1 + K_{cpx}\,[RH^+]}\right) + \log K_{ex} \tag{1.7}$$

and

$$\log K_{ex} = \log D_{Cl} - \log\left(\frac{[RH+]}{1 + K_{cpx}\,[RH+]}\right) \quad . \tag{1.8}$$

This equation is valid for low-loading. Provided that all of the molecules of extraction agent are protonated and there is not a large excess of chloride ion, it can be assumed that $[RH^+] = [R]$, a known quantity. Under these conditions, the equation simplifies to

$$\log K_{ex} = \log D_{Cl} - \log\left(\frac{[R]}{1 + K_{cpx}\,[R]}\right) \quad . \tag{1.9}$$

As is evident from the final equation (Eqn. 1.9), the equilibrium extraction ratio is dependent on the concentration of the carrier species, and the binding affinity of this agent for chloride.

1.4.4 Analysis with Radiotracers

Radiotracer analysis provides a highly sensitive method for the detection of analyte species. The technique, although not routinely available, has the advantage of being simple, and allowing direct measurements of partitioning ratios. Sulfur-38 (^{38}S) and Chloride-36 (^{36}Cl) are both beta-emitting species, releasing high-speed electrons as the isotopes decay. The half-lives of these two isotopes are 90 days and 301,000 years, respectively. Detection is very sensitive, necessitating only a very minute amount of radiotracer spiked into the bulk aqueous salt solution. Generally, 0.2 μCi/mL of radiation or less may be used in any given extraction experiment.

1.4.5 Instrumentation

The two-phase extraction experimental procedure is described in detail in Chapter 3. Briefly, equal amounts of an organic solution containing the extraction agent and an aqueous solution containing the analyte of interest (chloride ion in these experiments) are mixed on a rotating wheel at constant temperature until equilibrium is attained. After one hour, it is assumed that the extraction has reached equilibrium, the phases are separated and the relative amounts of chloride in each of the two phases are determined using beta counting. The original aqueous phase prior to extraction contained a solution of NaCl spiked with a small amount of $Na^{36}Cl$ or $H^{36}Cl$ radiotracer. After equilibrium is reached, the amount of radioactivity in each phase is proportional to the total concentration of chloride ion in each phase.

The detection of the radiotracer is made by measuring the light emitted by each beta particle as it is ejected. While a photon of light is emitted with each beta particle, due to scattering, those photons alone would not all reach the detector, resulting in a very low signal-to-noise ratio. To obtain a better signal, an aliquot of each phase is added to a

vial containing a mixture of xylenes before analysis. This "scintillation cocktail" amplifies the signal via energy transfer from the sample to the aromatic xylenes. Subsequent light-emission upon relaxation of the excited state allows for easier detection and better signal-to-noise ratios. The organic phase was subsampled at five times the volume of the aqueous subsample (250 uL vs. 50 uL) in order to compensate for the overall lower concentration of chloride, and specifically the lower concentration of ^{36}Cl, in the organic phase relative to the aqueous phase. A ratio of the counts from the organic aliquots relative to the corresponding aqueous ones were then plotted against the concentration of extraction agent on a log-log graph. The slope of the resulting line is taken as the distribution coefficient (D-value) and is considered proportional to the partition coefficient for the extraction process.

1.5 TRANSPORT STUDIES

While a great deal of information regarding the potential carrier molecules can be obtained using binding studies and partitioning experiments, these techniques do not replace experiments using true phospholipids bilayers and ultimately cellular systems. To that end, transport experiments using liposome models were employed.

Living biomembranes are composed of a myriad of various lipids, steroids, protein rafts and protein channels. The degree of complexity of a biomembrane structure with its various potential mechanisms for transporting materials in both directions presents a difficulty for analytical chemists. The goal of this work is to determine how well the carrier molecules under investigation mobilize chloride *on their own* and quite unassisted by protein channels and active pump mechanisms. Inasmuch as our scientific question is to determine the ability of these carriers to overcome the energetic barriers involved in moving an ion from a solvated, aqueous environment into a hydrophobic

environment in the absence of active assistance, it is logical to develop a model system in which other transport mechanisms do not exist. In this way, we eliminate competing mechanisms and reduce the question to its simplest form; that being, how well do these carriers transport chloride *unassisted* across a bilayer?

1.5.1 The Cell Membrane

All living organisms, however great or small, possess a cell membrane. The sequestering of necessary materials by the earliest forms of life from the "primordial soup" was likely one of the earliest events in evolution.[30] Our primitive ancestors likely had primitive cell membranes. In all probability, these were little more than a non-polar layer separating an inner watery compartment from the outerlying watery matrix.[31]

The oldest membrane-like material known was found in the Murchison meteorite, which was discovered in Australia in 1969. The meteorite was found to contain amino acids and a carbon compound which formed lipid-like droplets upon addition of water. The carbon material was dated at approximately 4.5 billion years old. At least another billion years would pass before true membranes existed around our primitive ancestors.[32]

1.5.1.1 Membrane biophysics

The modern eukaryotic cell membrane takes the form of a lipid bilayer, composed of two amphoteric leaflets, in which the polar headgroups orient themselves outward towards the inner and outer aqueous phases while the nonpolar chains are driven inward together, and away from the aqueous layers, by hydrophobic forces.[31] While the individual attraction between hydrophobic groups is small, as a result of the large number of lipids present in a bilayer, the overall cohesion is remarkably high.[31] In a typical

eukaryotic cell, there is an overall surface charge of approximately 1-100 mCm^{-2} due to an order of magnitude difference in the number of anionic groups versus cationic groups on the surface. Needless to say, the surface charge significantly influences the kinetics of ion transport across the membrane.

The primary components of any living animal membrane consist of phospholipids, such as phosphatidylcholine and sphingomyelin, steroids, and proteins. Due to the large percentage of mixed lipids, at physiological temperatures, most bilayers are fluid. Higher amounts of cholesterol in some membranes significantly restrict the fluidity of the bilayer.[33]

A lipid bilayer may exist in either of two distinct phases, the L-alpha liquid crystalline phase or the L-beta crystalline phase, depending on the temperature and the lipid components. The liquid crystalline phase exists above the transition temperature for the particular set of lipids comprising the membrane. In this phase, the acyl groups are gauche to each other and the nonpolar interior is highly fluid. The surface area of the membrane is increased and the thickness decreased, resulting in an increased volume compared to the L-beta crystalline phase of ca. 1-8%. Below the transition temperature, in the L-beta crystalline phase, the acyl groups are trans to each other, allowing the lipids to be packed tightly together and the membrane to be highly ordered.[32]

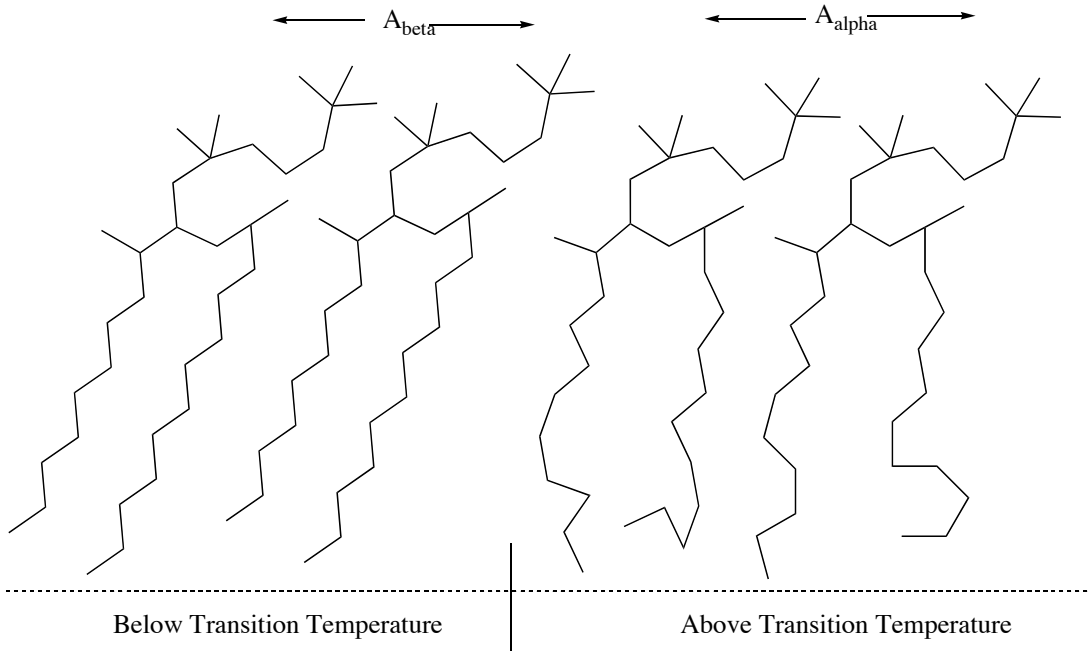


Figure 1.2 Schematic representation of the α-liquid crystalline and β-crystalline phases.

Figure 1.3 shows the various packing regions that exist in a lipid bilayer. The polar headgroups form a highly charged and freely rotating wall and the first line of defense against ion diffusion. To enter the interior of the membrane, an ion must first overcome the like-charge repulsion that is encountered at the membrane surface. Deeper into the membrane, the second barrier encountered by an ion is the region of tightly packed alkyl chains and cholesterol molecules present directly behind the headgroups. There is little in the way of charge-stabilization in this region and there is additional steric hindrance due to tight molecular packing. Beyond this region, in the innermost interior of the membrane, packing restrictions are alleviated, though the hydrophobicity of the medium is greatest.

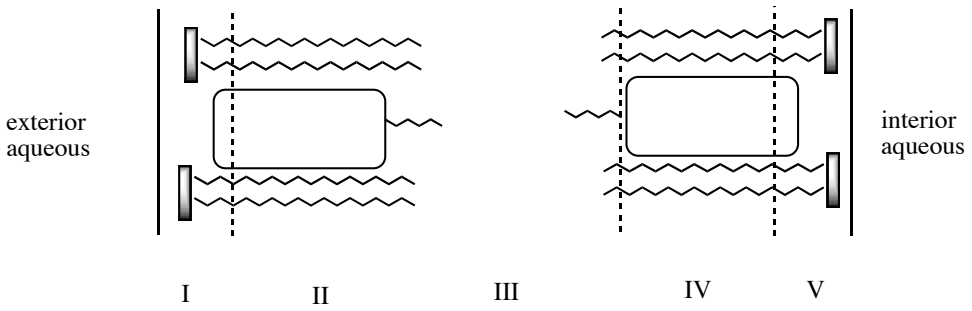
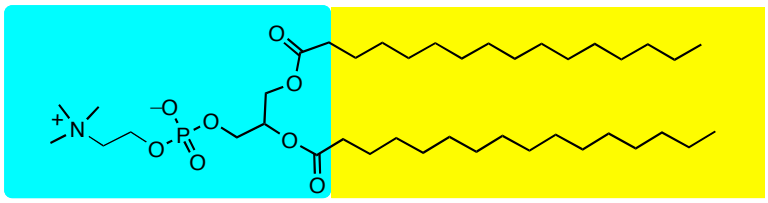


Figure 1.3 Polarity regions of a typical lipid bilayer.

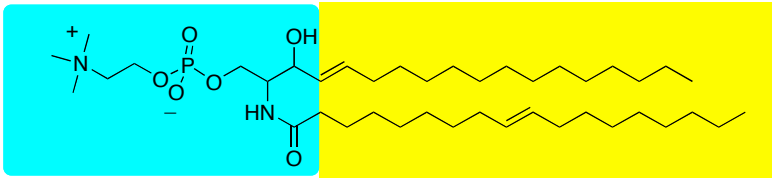
1.5.1.2 Components of a membrane

A living biomembrane is primarily composed of phospholipids, though there are a number of glycolipids present as well, which form the basic fluid bilayer. The majority of cellular phospholipids are derived from a glycerol backbone, such as phosphatidylcholine shown below (**1.5**). The polar headgroup consists of the esterified glycerol backbone, the phosphate group, and the choline group. The hydrophobic chains are of varying length and degree of saturation depending on their particular origins.



(1.5)

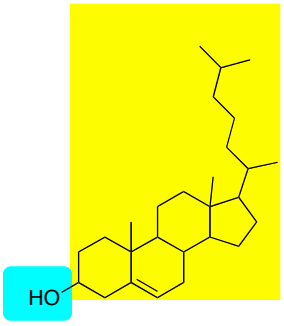
Not all phospholipids are derived from glycerol, however. Sphingomyelin (**1.6**) contains a sphingosine unit in place of glycerol and is very common in plasma membranes.



(1.6)

Sphingomyelin also possesses a phosphocholine unit in the headgroup, however, the backbone uses an amino alcohol and the hydrophobic tail is always unsaturated.[31]

Embedded in both leaflets of the bilayer are cholesterol units (**1.7**), which float in the bilayer, earning them the nickname "cholesterol rafts". Cholesterol is situated in the packing gaps between lipids, conferring additional stability by limiting the rotation of the lipid tails. The majority of the cholesterol molecule is hydrophobic and remains in the interior of the bilayer. The alcohol group is able to interact with water despite the overall hydrophobicity of the molecule and remains associated with exterior of the bilayer.



(1.7)

Additionally, the membrane has various types of proteins, some of which span the membrane to form channels, while others remain in either the outer or inner leaflet to act as receptors. These two scenarios are depicted in Figure 1.4.

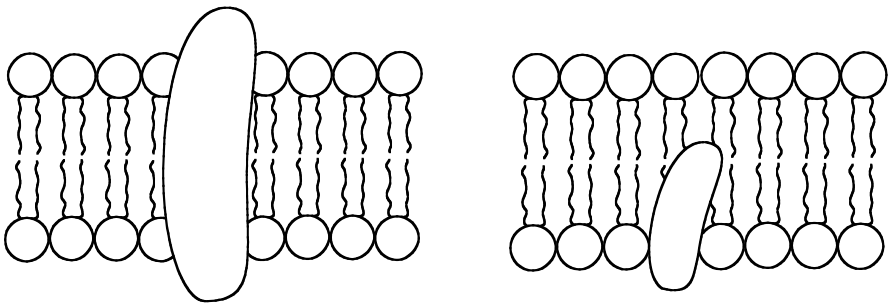


Figure 1.4 Schematic representation of two common interactions of proteins with a lipid bilayer. Both membrane-spanning and surface-associated proteins are shown.

Given the complexity of a living biomembrane, model studies have particular appeal. Especially for supramolecular chemists interested in studying carrier-mediated transport processes.

1.5.2 Liposomes

Liposomes provide a ready alternative to live cells for the study of membrane transport processes. Liposomes are spherical lipid bilayers that spontaneously form upon the hydration of various lipids. Initially multilamellar vesicles (MUVs) form upon hydration. The crude MUV suspension can be subjected to sonication or extrusion to yield a uniform liposome suspension of small, unilamellar vesicles (SUVs). Essentially, they are empty cells the size of which is highly tunable to the needs of the researcher.

1.5.2.1 Liposomes as membrane models

Liposome models present a useful method for studying transport due to their simplicity. They possess no protein channels and have no active transport mechanisms. There are some negative aspects to working with liposomes. Liposomes are generally less stable than living cells. Even when stored in refrigeration, liposomes are stable for a maximum of three to four days.[34] This lifetime varies with the exact composition of the bilayer, but three to four days is typical. Additionally, liposomes have "leakier" membranes than a living cell. This reflects the fact that liposomes are synthetic constructs make in general from one or two types of lipids. They thus lack the molecules that are typically used by a living cell to fill in the gaps in lipid packing are absent in liposomes. Some research groups compensate for this absence by adding cholesterol to their liposome structures. Cholesterol serves to limit the rotation of the lipids in the bilayer. By preventing the headgroups from freely rotating, cholesterol limits the ability of anions to directly cross the bilayer by anion perturbation. However, the use of cholesterol presents its own problems, which include membrane impermeability and excessive rigidity. This can adversely affect the basic extrusion process used to make liposomes. For these reasons, cholesterol was not added to any of the liposomes models used in this work.

1.5.2.2 Components of liposome bilayers

The phospholipids selected for the present studies were chosen on the basis of practicality. Two types of phosphatidylcholine, a synthetic and a natural version, and two types of phosphatidylserine, a synthetic and a natural version, were selected as the lipids of choice. L-α-phosphatidylcholine, extracted from egg yolks, is available as a mixture of phosphatidylcholines with varying alkyl chains. The percentages of distinct fatty acid

chains present in a typical egg yolk extraction mixture are as follows: 34.0% (16:0), 1.7% (16:1), 11.0% (18:0), 32.0% (18:1), 18.0% (18:2) and 3.3% (20:4), where the first number in parentheses is the length of the alkyl chain and the second number in parentheses is the number of double bonds in that chain.[34] The other phosphatidylcholine starting material is a synthetic analogue of natural phosphatidylcholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). It was purchased commercially. Natural L-α-phosphatidylserine, extracted from brain tissue, was also used. This material is a mixture of phosphatidylserines with varying alkyl chains. The fatty acid content of a typical brain extract mixture of phosphatidylserines is as follows: 33.0% (16:0), 7.3% (18:0), 4.3% (18:1), 46.9% (18:2), 7.1% (18:3) and 1.4% (unspecified). The specific synthetic analogue of phosphatidylserine used was 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS). As their names imply, the synthetic lipids are derived from palmitic acid and oleolic acid (16:0 and 18:1 for both POPC and POPS).[34]

The advantages of POPC and POPS and their natural extract siblings are that these lipids are relatively inexpensive and easy to manipulate. The transition temperature for these lipids, below which a less fluid, crystalline phase dominates, lies below room temperature (-18°C and -11°C, for POPC and POPS, respectively).[34] Because they remain in the fluid phase at room temperature, no special pains need to be taken to keep these lipids fluid. With other starting materials, it is often necessary to manipulate the lipids at elevated temperatures in order to ensure that all of the potential membrane components are incorporated into liposomes, rather than remaining as a semi-solid in the bottom of the flask.

1.5.3 Carrier considerations

A living cell possesses a number of mechanisms for mobilizing materials, ranging from passive diffusion through the bilayer itself to active transport via multi-protein relay networks.[30] Figure 1.5 highlights these different mechanisms. The only presumed relevance to these studies is that of carrier-mediated passive diffusion.

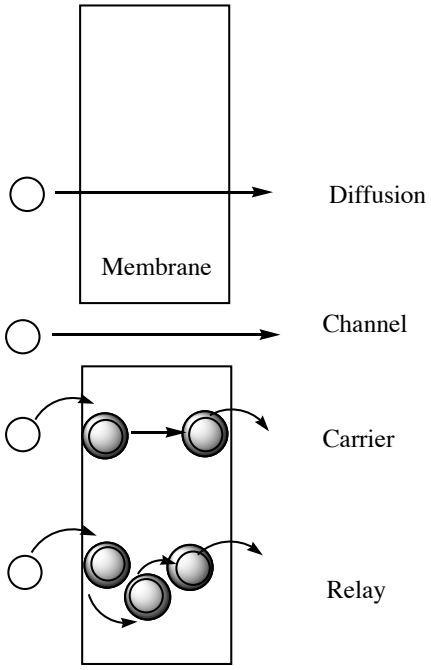


Figure 1.5 Mechanisms of transport in a living cell.

1.5.3.1 Ways of viewing a carrier

Carriers can be viewed in a several ways. An early conception of a carrier is depicted in Figure 1.6. In this scenario, the carrier initially exists in the aqueous phase and binds the desired species in the aqueous phase. The associated complex then crosses the membrane, enters the receiving aqueous phase, at which point the complex

dissociates in the aqueous phase. After the free carrier re-diffuses back through the membrane, the cycle repeats itself.

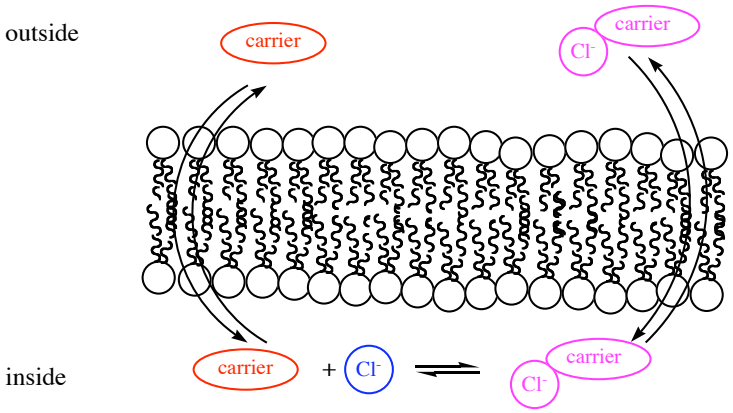


Figure 1.6 Early model of carrier-mediated diffusion shown with chloride anion as the translated species. Here, the carrier exists predominantly in the aqueous phase.

A more recent description of carrier-mediated diffusion envisions the carrier as being fully partitioned into the membrane,[22,30,33] as shown in Figure 1.7. In this scenario, anion binding occurs at the membrane surface, rather than in bulk solution. The binding is thought to be somewhat weak in aqueous solution, with stronger binding occurring as the complex diffuses through the membrane. Weak binding at the opposite surface results in the dissociation of the complex, completing the transfer event. In analogy to how an enzyme catalyzes a reaction via binding the transition state of the reaction more tightly than either the starting material or product, a carrier can be seen as a host that binds an anion more tightly in an intermediary hydrophobic environment than in the surrounding aqueous environments.

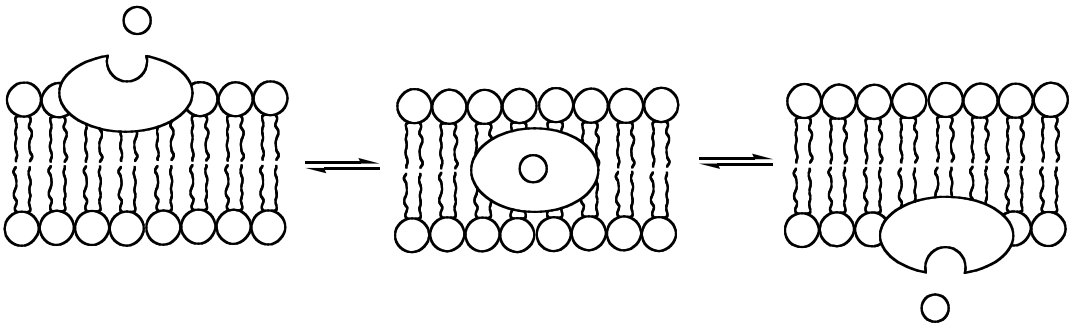


Figure 1.7 A modern description of carrier-mediated diffusion. The carrier is fully partitioned into the membrane with strong binding occurring in the hydrophobic interior and relatively weak association/facile dissociation occurring at the membrane surfaces.

1.5.3.2 Lipophilicity

Considering the modern description of carrier-mediated diffusion, a transport agent needs to be sufficiently lipophilic so as to remain partitioned into the interior of the bilayer. A certain amount of water-solubility is necessary for the studies, however. Should a solution of transport agent crash out as a solid upon addition to the aqueous liposome suspension, little or no carrier will partition into the membrane and no transport will occur.

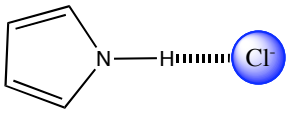
1.5.3.3 Charge-neutralization

Assuming that naked ions will not exist as isolated charges in the hydrophobic interior of a bilayer, it is reasonable to imagine that a positively charged carrier would effect anion transport more readily. The resulting ion-paired complex would have no net charge, be able to diffuse across the membrane interior, and readily dissociate upon contact with a second aqueous environment. The anion concentration difference in the

two aqueous phases (inside and outside of the membrane) would provide the overall driving force for the process.

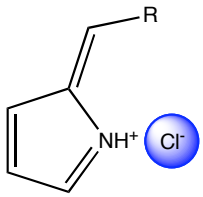
1.6 THE PYRROLE GROUP

Research in the Sessler Group centers around pyrrole chemistry and its anion-binding behavior. Linear molecules and macrocycles derived from pyrroles have been developed over the years to recognize and bind various anions selectively, including chloride. As a hydrogen-bond donating molecule that is easy to functionalize and incorporate into larger structures, pyrrole represents a very attractive "building block" for the design of effective chloride extraction and transport agents.[35-44]



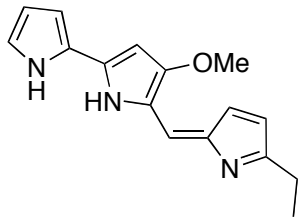
1.6.1 Protonation state

Oxidized pyrrole macrocycles and linear pyrrole molecules possess imine-like nitrogen atoms that readily protonate.[45-49] These protonated species would be capable of ion-pairing to form neutral complex for transport as discussed in Section 1.5.3.3.



1.6.2 Prodigiosins

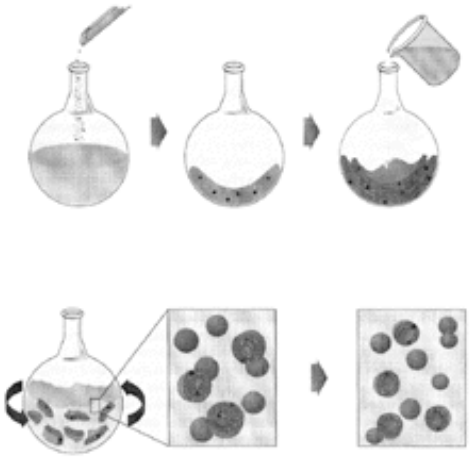
The prodigiosins are a class of pyrrole-based natural products that have been implicated in HCl symport[50-51] across cell membranes and which possess recognized cytotoxic behavior.[52-55] This class of molecules has served as inspiration for a new generation of linear pyrrole-based molecules developed in the Sessler Group with potential application in disease treatment. They have also served as a springboard for the design of new oligopyrrolic molecules with potential anion transport capabilities.



The natural prodigiosins are derived from the basic scaffold shown above. Derivatives of these prodigiosins have the advantage of allowing various structural functionalities to be examined individually, providing he basis for designing future generations of presumably more efficient transport and extraction agents.

1.7 INSTRUMENTATION

Liposome suspensions are prepared by hydrating a dry lipid film with agitation. The initial hydration results in a complex mixture of multilamellar liposomes of varying sizes, as shown in Scheme 1.5 below.



Scheme 1.5 Preparation of liposome suspension. Hydration of the lipid film results in a crude LUV suspension. Repeated extrusion through a polycarbonate membrane supplies a uniform SUV suspension.

This mixture must be reduced to a suspension of unilamellar vesicles of uniform size. There are several techniques for preparing a uniform vesicle solution, including vigorous sonication. Sonication generally results in small, unilamellar vesicles (SUVs) with a mean diameter, d, of less than 50 nm, a size too small for the studies described in this dissertation. Extrusion through a polycarbonate membrane, whose pore size corresponds to the desired diameter of the SUVs proved to be the method of choice for obtaining liposomes appropriately sized (d = 200 nm) for these studies.

1.7.1 Mini-extruder for liposome preparation

The basic setup for liposome extrusion consists of the extruder and two syringes. More elaborate setups, such as the one shown in Figure 1.8, include a mounting/heating block into which a thermometer can be fit. The entire apparatus can be placed on a hotplate so that extrusion can be performed at elevated temperatures for those lipids

whose unfavorable phase transition temperatures necessitate such accommodations. The polycarbonate membrane is placed between two Teflon plugs with filter supports on each side. The Teflon plugs, as well as the supports and membrane, are then placed into a nut-and-bolt container, which is screwed tightly together in order to prevent loss of material. Finally, a crude liposome suspension is taken up into one syringe, which is then inserted into one end of the extruder; the empty and fully plunged syringe is inserted into the opposite end. The liposome suspension is passed through the membrane by alternately depressing each syringe until a uniform suspension is obtained. Avanti Polar Lipids Inc. suggests a minimum of 11 passes through the membrane in order to ensure a uniform suspension. In these studies, liposome suspensions were extruded no less than 39 times through the polycarbonate membrane.

Figure 1.8 Extrusion of a crude LUV suspension. A mini-extruder mounted on a heating block is shown for the process.

1.7.2 Chloride-selective electrode

Transport into or out of a liposome can be studied by monitoring the accumulation inside or the loss from the interior of the liposome. These experiments are referred to as influx or efflux experiments, respectively. In the cases of the molecules under investigation, efflux experiments were found to be the most reliable and convenient. Figure 1.9 illustrates the basic principle of an efflux experiment.

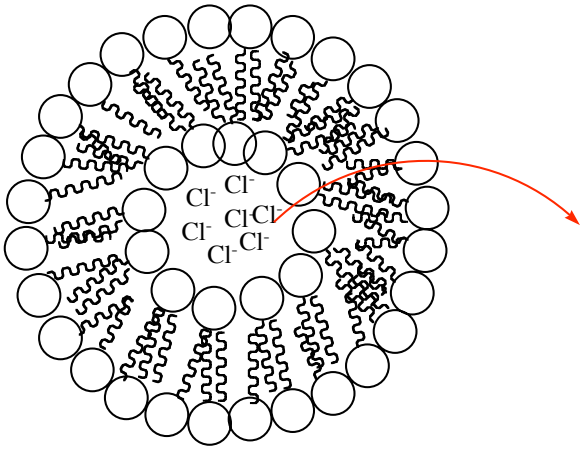


Figure 1.9 Chloride efflux from a liposome.

The experimental setup and instrumentation for the transport experiments consisted of a vial containing a liposome suspension (5 mL, 1mM lipid concentration) equipped with a stirbar, and placed on a stirplate. A chloride-selective electrode was inserted into the suspension. The liposomes contained 500 mM NaCl inside the vesicles. The outer solution, in which the vesicles were suspended, consisted of a 500 mM $NaNO_3$, 5 mM buffer (TES for MES) solution. After obtaining a basal reading, which was taken to be 0% chloride release, aliquots of carrier were added via microsyringe. The electrode reading was monitored over a period of five minutes as the carrier transported chloride

from inside the vesicles to the outer solution. After five minutes, the liposomes were lysed to release all of the chloride. After this, a final reading was taken that was defined as being 100% chloride release. The experimental apparatus is shown in Figure 1.10.

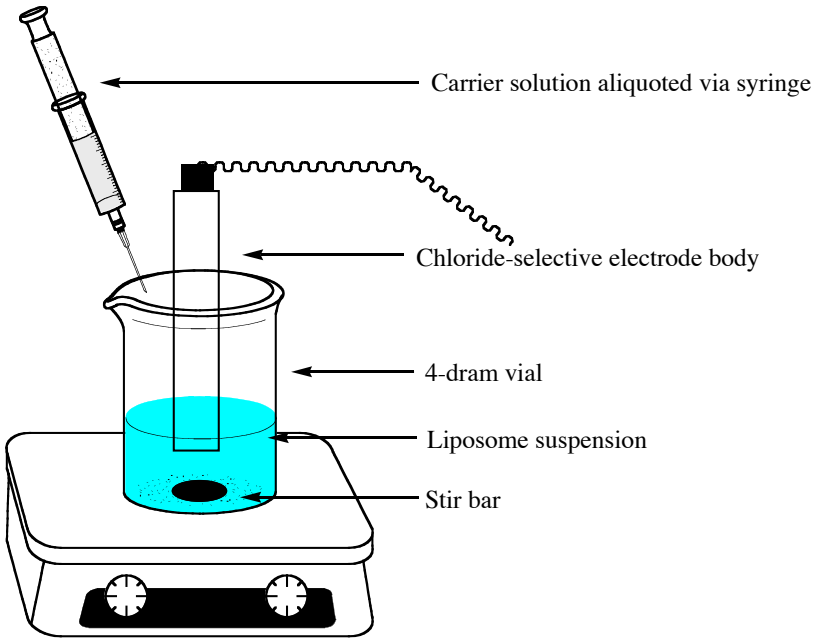


Figure 1.10 Chloride-selective electrode setup.

Commercially available chloride-selective electrodes are based on the potential difference across a silver chloride membrane that develops as chloride ions interact with the membrane. This difference is proportional to the activity of the chloride ions and is described by the following equation.

$$E = E° - S \log A$$

where E = measured electrode potential, E° = standard potential, S = electrode slope and A = chloride activity.

At 25°C, the Nernstian slope is -59.2 mV per decade increase in chloride activity. Calibrations were performed manually using 100 mL NaCl solution standards with concentrations ranging from 1 ppm to 10,000 ppm (10^{-6} – 10^{-1} M), with measurements taken every decade. To each standard solution, 2 mL of Ionic Strength Adjuster™ (ISA)—a 5 M $NaNO_3$ solution—were added. The response time of the electrode used is relatively quick. Stable readings can be obtained in less than 10 seconds at most concentrations.

1.8 SUMMARY AND OUTLOOK

The chapters to follow discuss three research projects that were completed for this dissertation. Each project is distinct and could be seen to stand alone; anion binding, anion extraction and anion transport are all individual areas of current research. Taken together, these projects address the fundamental science that is common to all. Chapter 2 describes the sulfate-extraction behavior of certain macrocycles developed in the Sessler Group. Chapter 3 delves into the chloride-extraction behavior of a number of pyrrole-based compounds. Both protonated and neutral species are examined and compared in these studies. Chapter 4 explores the carrier-mediated diffusion behavior of those pyrrolic molecules that exhibited favorable anion-partitioning behavior in the previous chapter. The common goal of all three projects was to understand the determinants that might allow for the design of pyrrole-type receptors that are able to surmount the

energetic barrier to the movement of anionic species from an aqueous source phase into or through a hydrophobic environment.

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Chapter 2: Liquid-Liquid Extraction of Sulfate

2.0 INTRODUCTION

Hefter has written an extensive review on the energetics of the transfer of electrolytes into organic media.[1] There are a number of detailed texts on the topic of liquid-liquid partitioning and equilibria to which the reader is also referred.[2-5] A thorough discussion of the physical chemistry of partitioning events is beyond the scope of this dissertation.

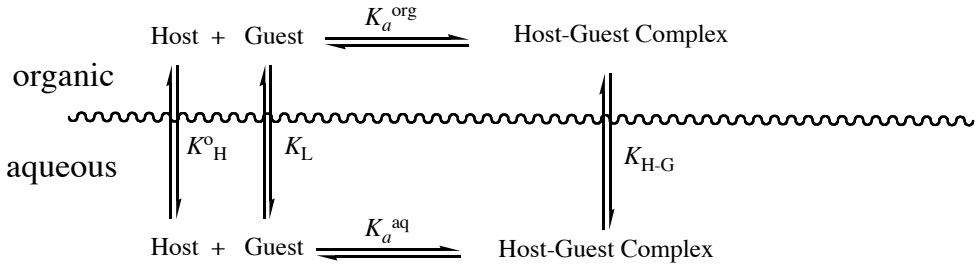
Liquid-liquid partitioning has been used extensively in separations science, and it is in this arena that the present work will focus. To a lesser extent, partitioning has been used to measure binding constants and other molecular characteristics, such as hydrophobicity. Commonly, it is exploited in organic synthesis workups. Little attention is given to the partitioning coefficients of the solutes directly, however every organic chemist knows that extracting an aqueous solution with several volumes of solvent in a separatory funnel will deliver them more of their desired product. Partitioning behavior influences good scientific results even when it is not the experimental question!

2.1 GENERAL CONSIDERATIONS

In order to achieve an efficient extraction, the overall process must be carefully considered and the actual experiments designed appropriately. The extraction agent should remain entirely partitioned into the organic phase; thus the desired agent should be strongly hydrophobic. Carriers that partition into the aqueous solution are of course removed from the organic phase, reducing the concentration of carrier available to

perform extractions and decreasing the efficiency of the entire process. A second consideration is electrical neutrality. Ions will not partition favorably into organic solution as charged species since this would result in a net buildup of separated charges in each of the two phases. Unless the carrier species itself can readily act as the counterion, a second species, a co-extractant, must be used.

As discussed in Chapter 1, partitioning is an equilibrium process and though the kinetics of extraction are sometimes important, in general it is equilibrium considerations that dominate the separation process. Thus, thermodynamic studies remain the focus of most current work. Given this, the relevant equilibria that govern possible extraction processes must be considered.



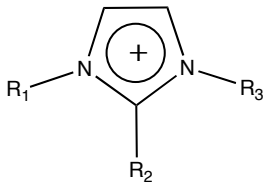
Scheme 2.1 Extraction equilibria.

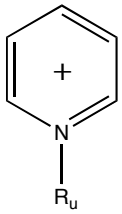
2.2 PREVIOUS WORK

Liquid-liquid extraction has been exploited to separate various types of analytes. Ions having similar sizes and shapes are particularly difficult to separate from one another using this technique. In these cases, small differences in molecular identity must be exploited in order to affect separation. The Moyer group has considerable experience in dealing with this challenge since the cations of interest to these researchers, namely the alkali metals cesium and francium, fall into this category. Being metal cations, these ions

have similar spherical shapes. Further, being located in the 6th and 7th periods of the periodic table, respectively, both ions have very large ionic diameters. However, by using a crown-calixarene compound[7] previously considered to be Cs^+ selective, the Moyer group was able to effect significantly higher extraction of Fr^+ from an aqueous $NaNO_3$ solution than Cs^+ from a similar matrix. The molecule showed higher selectivity for francium ions.

Turner and coworkers have utilized liquid-liquid partitioning to examine the hydrophobicities of various common metal ion pollutants.[8] Other groups have exploited the inclusion capacities of several calixarenes, combining their chemistry with ionic liquids, in which the solvent molecules themselves are charged species, such as the two shown below, to extract ions from aqueous solution.[9]





Hydrophobic ionic liquids are solvents that bear a charge and yet are insoluble in water due to their "greasy" alkyl groups. Such media have been shown to provide a ready counter-ion for extraction anions, thus eliminating the necessity for a co-extractant such as Aliquat 336. Using an ionic liquid based on imidazolium and pyridinium, such as are shown above, it has been found that the calixarene selectively removes cesium cations, while the solvent acts to stabilize the counter anion brought in from the aqueous phase.

2.3 SULFATE AS AN ANALYTE

Nuclear waste remediation is a daunting, yet necessary task facing modern society. The Yucca Mountain Project is an effort to re-locate radioactive wastes from current facilities, which are near inhabited areas, and store them deep inside Yucca Mountain. The Westinghouse Hanford waste tanks present one of the most challenging targets for waste clean-up and relocation. The radioactive waste is a complex mixture of radioactive and cold materials, salt caches and sludge. As such, the waste is difficult to treat. Extraction-based separations could provide a much-needed means of addressing this cleanup problem.[10] In particular, such an approach might allow the components to be isolated and dealt with individually, thus simplifying cleanup efforts and moderating procedural costs.

The Department of Energy (DOE) has long been of the opinion that by vitrifying the salt caches and removing the risk of a spillage, the radiation can be rendered much less hazardous. A difficulty with the vitrification process is the sulfate component of the salt mixture. By far, one of the most abundant ions in the salt caches is nitrate, which can be converted into glass fairly readily. A typical tank summary (Tank 241C-101) is shown in Table 2.1. Sulfate contamination prevents the vitrification process from taking place as efficiently or as cost-effectively.[10] Sulfate, therefore, presents an attractive target for the development of selective extraction agents. Upon the removal of sulfate, the remaining mixture can be vitrified, sealed in protective casing and stored with a greatly reduced risk of a contamination leak.

Single-Shell Tank 241-C-101			
TLM Solids Composite Inventory Estimate			
Physical Properties			
Total Solid Waste	4.74 E +05 kg	(88.0 kgal)	
Heat Load	2.26 E -02 kW	(77.1 BTU/hr)	
Bulk Density	1.42 (g/cc)		
Void Fraction	0.825		
Water wt%	55.9		
TOC wt% C (wet)	9.59 E -05		
Chemical Constituents	**mol/L**	**ppm**	**kg**
Na^{+}	2.41	3.89 E +04	1.85 E +04
Al^{3+}	3.42	6.48 E +04	3.07 E +04
Fe^{3+}	0.727	2.85 E +04	1.35 E +04
Cr^{3+}	2.58 E -03	94.3	44.7
Bi^{3+}	0	0	0
La^{3+}	0	0	0
Hg^{2+}	2.54 E -03	358	170
Zr (as $ZrO(OH)_2$)	0	0	0
Pb^{2+}	0.133	1.93 E +04	9.15 E +03
Ni^{2+}	1.29 E-03	53.2	25.2
Sr^{2+}	0	0	0
Mn^{4+}	0	0	0
Ca^{2+}	0.253	7.11 E +03	3.37 E +03
K^{+}	6.32 E -03	174	82.4

OH^-	14.3	1.71 E +05	8.11 E +04
NO_3^-	1.09	4.73 E +04	2.25 E +04
NO_2^-	0.420	1.36 E +04	6.43 E +03
CO_3^{2-}	0.371	1.56 E +04	7.41 E +03
PO_4^{3-}	5.42 E -02	3.62 E +03	1.72 E +03
SO_4^{2-}	5.34 E -02	3.60 E +03	1.71 E +03
Si (as SiO_3^{2-})	5.82 E -03	115	54.4
F^-	0	0	0
Cl^-	3.63 E -02	902	428
$C_4H_5O_7^{3-}$	0	0	0
$EDTA^{4-}$	0	0	0
$HEDTA^{3-}$	0	0	0
$Glycolate^-$	0	0	0
$Acetate^-$	0	0	0
$Oxalate^-$	0	0	0
DBP	9.48 E -06	1.77	0.840
Butanol	9.48 E -06	0.494	0.234
NH_3	3.57 E -04	4.26	2.02
$Fe(CN)_6^{4-}$	0	0	0
Radiological Constituents			
Pu		0.566 (µCi/g)	4.47 (kg)
U	0.208 M	3.47 E +04 (µg/g)	1.65 E +04 (kg)

Cs	1.79 E -03 (Ci/L)	1.26 (μCi/g)	596 (Ci)
Sr	8.82 E -03 (Ci/L)	6.19 (μCi/g)	2.94 E +03 (Ci)

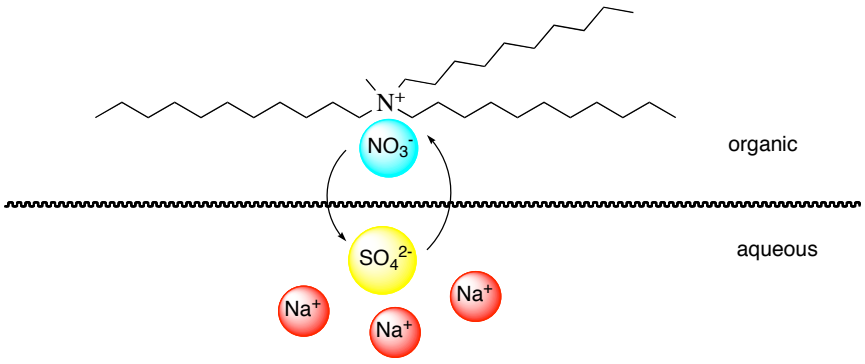
Table 2.1 Tank 241-C-101 Inventory of solids.[11] Unknowns in the tank solids inventory are assigned by Tank Layering Model. This data and others reports from the national laboratories are available for download from: http://www.osti.gov/bridge/search.easy.jsp

Ideally, an extraction agent would be completely selective for the desired analyte, in this case sulfate, in the presence of a variety of other anions. The needs of the DOE are such that remarkably high selectivity in the presence of several analytes with very similar structures and geometries is not as crucial. Rather, extraction agents are required that can successfully extract sulfate from actual tank waste (i.e. in the presence of a very high concentration of nitrate anion). The intended use reduces the stringency of criteria for an efficient extraction agent. Anions whose size and shape are the most similar to sulfate, such as phosphate, are not present in higher concentrations than sulfate in the target salt caches (see Table 2.1), thus the competition they represent is limited. As noted above, nitrate is present in concentrations that are several orders of magnitude higher than sulfate. It thus provides the greatest competition for binding. Fortunately, nitrate differs structurally from sulfate. Nitrate is a trigonal planar anion with a single negative charge. Sulfate is a tetrahedral analyte that is dianionic at neutral pH. On the other hand, the ion is large and charge-disperse, decreasing the efficiency of anion hydration, which favors the extraction process. Nonetheless in comparison with nitrate, sulfate is significantly more structure-forming, having a partial molal hydration entropy of only + 17 J/(K•mol). The partial molal hydration entropy for nitrate is +125 J/(K•mol). Being a harder anion than nitrate, sulfate is less stabilized in a nonpolar environment, meaning that extraction

of this anion is enthalpically unfavorable. However, removal of this structure-making anion from aqueous solution would lead to an increase in entropy, meaning that the extraction of sulfate should be an entropically favored process.

2.4 SULFATE EXTRACTION STUDIES

These studies were performed with two goals in mind. The first was to investigate the sulfate extraction ability of pyrrole-based macrocycles for potential use in waste remediation processes. The second goal was to learn how to perform radiotracer analyses, which would later be applied to chloride extraction (those experiments are dealt with in Chapter 3). In all of the sulfate extraction experiments associated with this dissertation work, the protocol was essentially identical. Along with experimental carriers, a co-extractant, Aliquat A336-nitrate (A336-NO_3 or simply, A336) was used. A336-nitrate is a lipophilic, quaternary ammonium nitrate compound often used in partitioning experiments. The ammonium nitrate species is co-dissolved with experimental carrier molecules in organic solvent. The nitrate anion is able to exchange with the desired anion, in this case sulfate, at the interface of the two phases, while the ammonium cation remains in the organic phase to provide the counterion for the extracted sulfate anion (see Scheme 2.1).



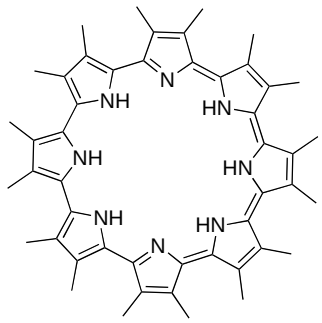
Scheme 2.1 Aliquat 336 nitrate-sulfate exchange mechanism. A336 provides the necessary counter-cation to maintain electrical neutrality in the organic phase. A336 is not expected to partition into the aqueous phase to any degree.

The extraction agent (A336 alone or A336 + carrier) was dissolved in chloroform or nitrobenzene, depending the solubility requirements of the molecule, and subjected to serial dilutions in a 1 mL centrifuge tube to give final organic volumes of 450 μL. In the radiation hood 450 μL of a spiked aqueous salt sample were added to each centrifuge tube. The tubes were sealed, checked for radiation leaks and placed on a rotating wheel. The phases were contacted for one hour at constant temperature until equilibrium was reached. The tubes were then returned to the radiation hood where the phases were separated and subsampled. Aliquots, consisting of the aqueous phase and organic phase (50 μL and 250 μL, respectively) were subsampled. The difference in sample volume reflected the lower amount of electrolyte, and hence reduced amount of detectable radiation, in the organic solution. Subsamples were added to scintillation vials filled with 5 mL of Ultima Gold™ scintillation cocktail. The vials were allowed to sit in the dark for approximately 30 minutes before placing them on the beta counter. Raw data counts were corrected for volume differences in the subsampled phases and background

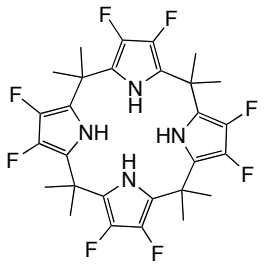
radiation counts. Distribution ratios were determined by dividing the number of corrected organic counts by the number of corrected aqueous counts, which is proportional to the total sulfate concentration in the organic phase divided by the total sulfate concentration in the aqueous phase.

The sulfate extraction data for two compounds (shown in Figure 2.1) developed in the Sessler Group are presented here. Fluorinated calix-pyrrole was provided by Dr. James A. Shriver and cyclo[8]pyrrole was provided by Dr. Thomas Koehler, both previous members of the Sessler group. Also presented are the control data for Aliquat 336-nitrate (A336). A336 demonstrates some ability to extract sulfate in the absence of any other carrier molecule. The ratio of carrier to guest is 2:1 (ammonium to sulfate), as would be expected in order to maintain charge balance. A control study was also performed to examine sulfate extraction by A336 (only) in the presence of increasingly high concentrations of nitrate.

It should be noted that the sulfate extraction studies were performed in part as learning tool in order to become familiar with radiotracer studies prior to performing the chloride extractions discussed in the following chapter. As such, fewer molecules were addressed in the sulfate extraction portion of this dissertation. Cyclo[8]pyrrole and fluorinated calixpyrrole were the molecules examined. They are shown in Figure 2.1.



Cyclo[8]pyrrole



F-Calixpyrrole

Figure 2.1 Macrocyles investigated for sulfate extraction. Cyclo[8]pyrrole and fluorinated calixpyrrole were tested for their ability to extract 0.1 mM Na_sSO_4 from a 10 mM $NaNO_3$ matrix.

2.4.1 A336 Control

Sulfate, being dianionic under the interfacial conditions tested, will associate with A336 as a 1:2 anion : ammonium cation complex of in the absence of another carrier so as to maintain electrical neutrality. The control experiments examined the efficiency of A336 to effect the extraction of sulfate in a nitrate matrix. In these experiments the concentration of Na_2SO_4 was held constant at 0.1 mM, while the concentration of $NaNO_3$ was varied from 5-40 mM. The resulting data are summarized in Figure 2.2. At each concentration of sodium nitrate solution examined, the R^2 values are very close to unity, indicating reliable curve-fitting. In all of the control experiments the association was 2:1 (A336 : sulfate anion), as expected. Also in accord with expectations, A336 was found to display little preference for sulfate over nitrate as indicated by the decrease in overall sulfate extraction with increasing nitrate concentration. The nitrate effectively competes with sulfate for the ion-pairing associations provided by A336.

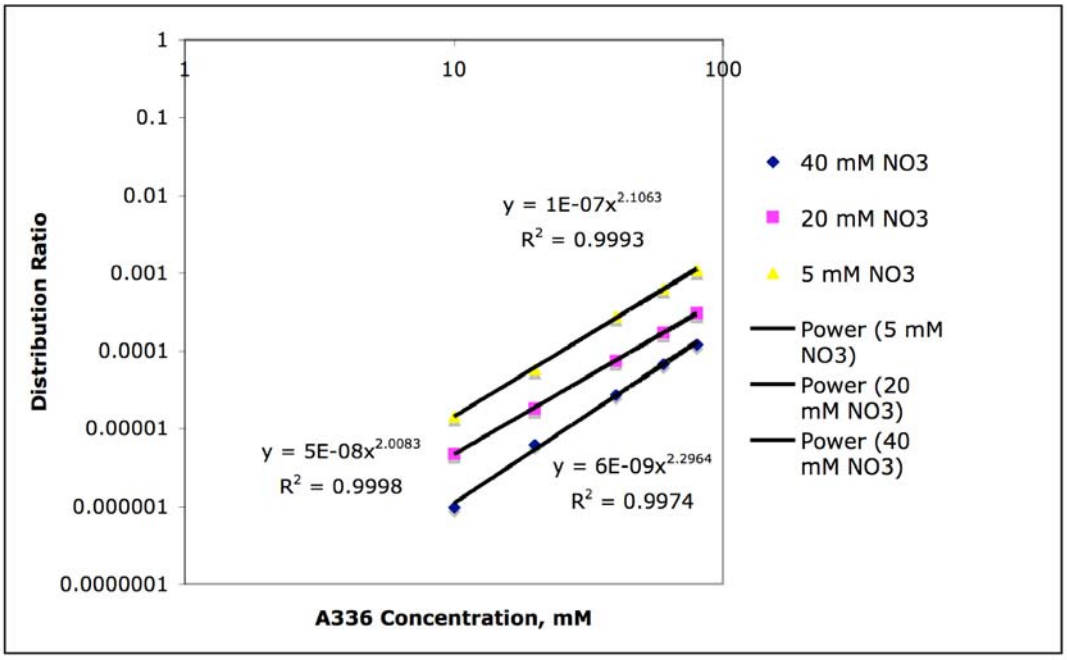


Figure 2.2 Sulfate extraction behavior of A336. Sulfate anions were extracted from solutions that contained 0.1 mM Na_sSO_4 and varying concentrations of $NaNO_3$. The trendlines and resulting line equations were determined using the software program Excel. As shown in the graph legend, the data trend fit a power equation.

A second control experiment was performed in which the nitrate concentration and the A336 concentration were held constant (1 mM and 10 mM, respectively) while the concentration of sulfate was varied. The data acquired from these studies are summarized below in Figure 2.3. Briefly, a decrease in the distribution ratios was seen once the sulfate concentration was allowed to increase above 3 mM, presumably reflecting saturation of the carrier.

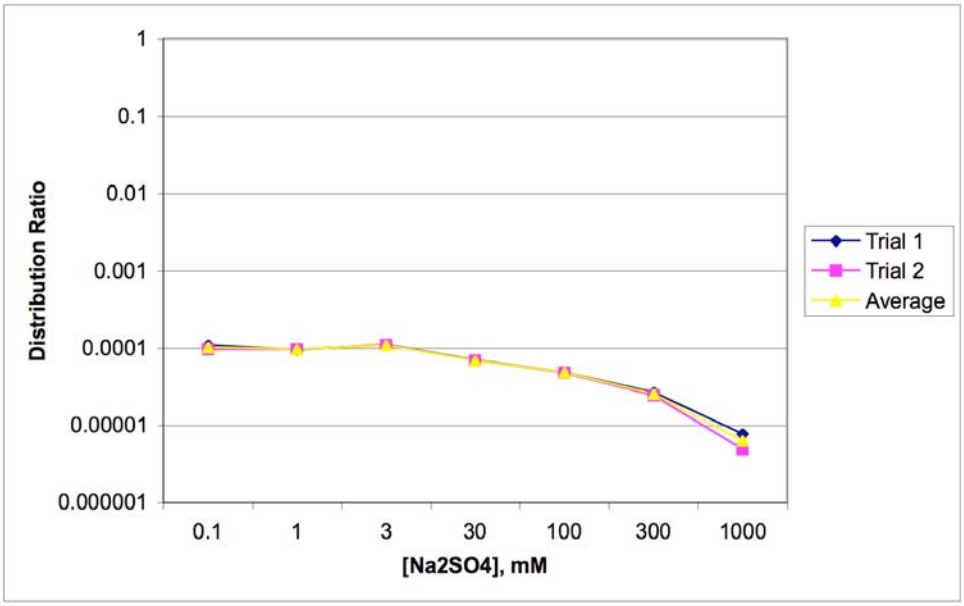


Figure 2.3 Extraction behavior of A336 with varying concentrations of sulfate. Saturation was evident above 3 mM Na_sSO_4.

2.4.2 Cyclo[8]pyrrole

Cyclo[8]pyrrole proved to be an extremely efficient sulfate-extraction agent. See Figure 2.4. At higher concentrations, distribution ratios approaching unity were obtained. The data fit the calculated curve extremely well; R^2 = 0.9963. The association of cyclo[8]pyrrole and sulfate was indicated to be a 1:1 complex, which corresponds to the anticipated sandwich complex of A336-sulfate-cyclo[8]pyrrole (Scheme 2.2). Since cyclo[8]pyrrole is presumed to be mono-protonated at the pH in question (ca. pH 7), an overall 2:1 ratio of binding species : anion is expected.

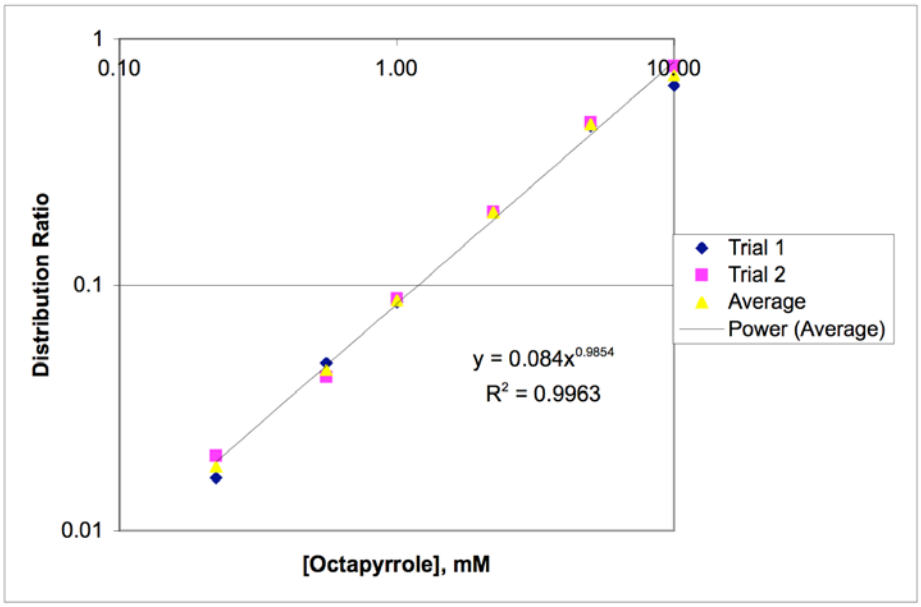
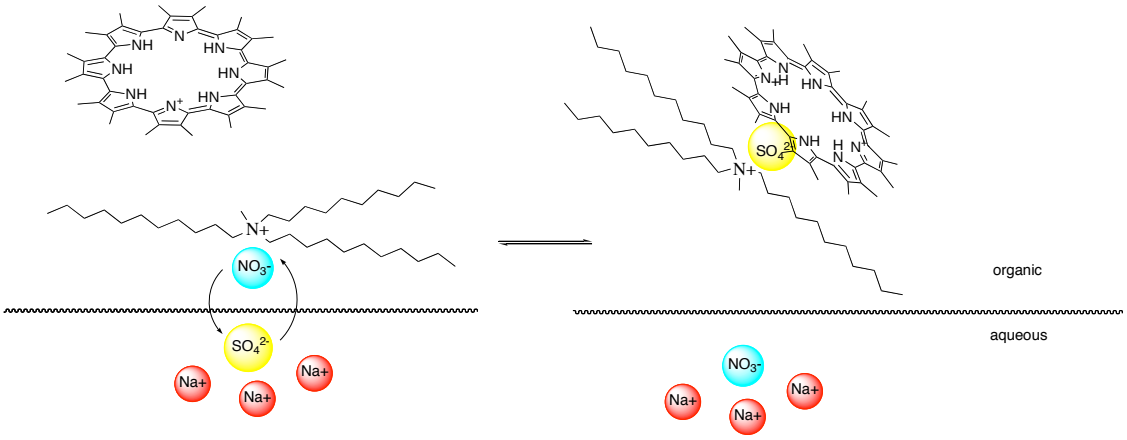


Figure 2.4 Sulfate extraction behavior of cyclo[8]pyrrole in a $NaNO_3$ matrix. Aliquat 336-nitrate was co-dissolved with 10 mM of the extraction agent to give a solution that was 80 mM A336 and 10mM carrier. Serial dilutions were performed from that stock solution. The trendline and resulting line equation were determined using the software program Excel. As shown in the graph legend, the data trend fit a power equation.



Scheme 2.2. Extraction Process. 1:1:1 Proposed ternary complex formed from dianionic sulfate, cyclo[8]pyrrole, and Aliquat 336. This is not expected to be the only species formed under the interfacial conditions of the extraction experiment. However, it is proposed as being one of several complexes responsible for the efficient extraction seen in these kinds of experiments.

2.4.3 Fluorinated calixpyrrole

Fluorinated calixpyrrole displayed some small amount of sulfate extraction as shown in Figure 2.5. The data did not fit a logarithmic or power curve. Because there was no reliable curve-fitting, no association stoichiometry could be inferred from the data. Given the smaller binding pocket of a calix[4]pyrrole, as compared to cyclo[8]pyrrole, it is not surprising that sulfate was not readily extracted by this species. Fluoride modifications to the beta-positions of pyrroles generally increasing the binding ability of those hosts. However, cavity size and the lack of an appropriate geometric match appears to be more important determinants; these factors preclude efficient sulfate extraction by calixpyrrole.

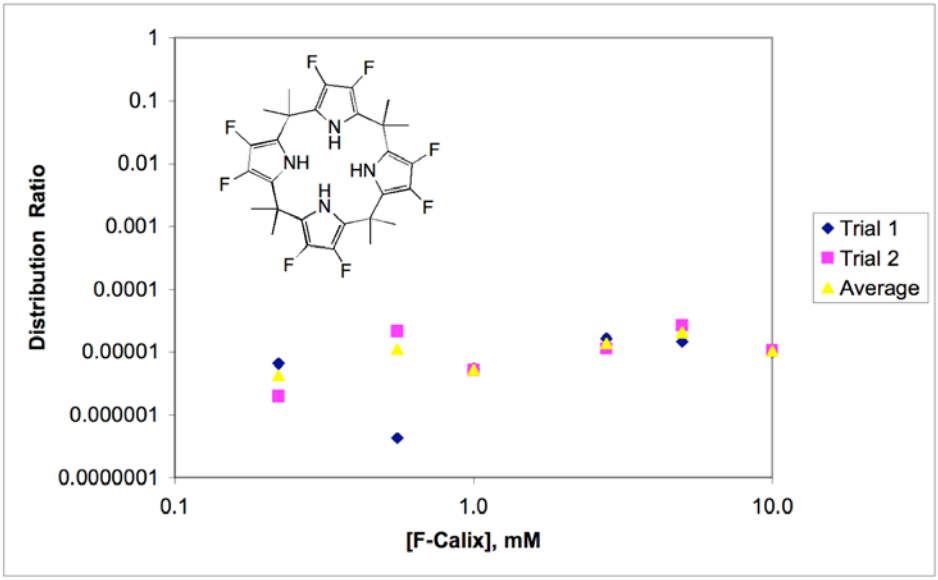


Figure 2.5 Sulfate extraction behavior of fluorinated calixpyrrole from a 10 mM $NaNO_3$ matrix. Aliquat 336-nitrate was co-dissolved with the 10 mM extraction agent (80 mM highest concentration). The second data point of Trial 1 was anomalously low and not reproducible.

2.5 Summary of Sulfate Extraction Experiments

To reiterate, the primary goal of the sulfate extraction experiments conducted and discussed in this dissertation was to develop an understanding of radiotracer techniques that could be later applied to the chloride extraction studies. Chlorine-36 is a strong beta-emitter, and a weak gamma emitter, with a long halflife (3×10^5 years), and is therefore much more dangerous in the hands of a novice. Sulfur-38 is a weak beta-emitter with a short halflife (90 days), and is therefore a much safer radiotracer with which to break in oneself.

Cyclo[8]pyrrole was shown to be a highly efficient extraction agent for sulfate and was shown to be very selective for sulfate over nitrate. The main drawback to cyclo[8]pyrrole was its sparse solubility in organic solvents. A second generation of

more soluble cyclo[8]pyrroles could prove very useful as an aid to effecting extraction-based radioactive waste remediation. Efforts to prepare such systems are currently being made by others in the Sessler group, notably Mr. Jeong Tae Lee and Dr. Wyeth Callaway.

2.6 EXPERIMENTAL

General considerations

Solvents were used from the bottle without additional drying. Aliquat A336-nitrate was kept under vacuum when not in use. Carrier molecules obtained from Drs. James Shriver and Thomas Koehler were dried under vacuum prior to use.

Sulfate partitioning experiments at Oak Ridge National Laboratory

In the control experiments using only A336, solutions were initially prepared at 80 mM concentrations in chloroform. To six 1-mL centrifuge tubes were added 10mM carrier solutions in chloroform or nitrobenzene (calixpyrrole and cyclo[8]pyrrole, respectively) in the following amounts: 450 μL, 225 μL, 100 μL, 50 μL, 25 μL and 10 μL. An additional volume of chloroform was added to each tube in the following amounts: 0 μL, 225 μL, 350 μL, 400 μL, 425 μL 440 μL, respectively. As a result, every centrifuge tube contained 450 μL of organic solvent and contained the carrier in question in the following concentrations: 10 mM, 5 mM, 2.2 mM, 1.1 mM, 0.6 mM, 0.2 mM, respectively. Similar dilutions of A336 alone with chloroform gave carrier concentrations of 80 mM, 60 mM, 40 mM, 20 mM, and 10 mM. In the experiments using A336 and another extraction agent, the two materials were co-dissolved. An initial solution of 80 mM A336 and 10 mM experimental molecule was prepared. Identical dilutions as described above were prepared. To each centrifuge tube, 450 μL of spiked aqueous sulfate solution were added in the radiation hood. The centrifuge tubes were then sealed, removed from the radiation hood, tested for radiation leaks, and placed into 50-mL conical vials packed with Kimwipes™. The conical vials were sealed and placed on a mechanical rotating wheel for one hour. The vials were then removed and the inner

tubes were centrifuged for 10 minutes to re-separate the layers. 50 μL of each aqueous layer were subsampled. 250 μL of each chloroform layer were subsampled. The subsamples were each independently added to 10 mL of scintillation "cocktail" and placed into a beta scintillation counter. From the counting data, distribution ratios were determined using the software program Excel.

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Chapter 3: Liquid-Liquid Extraction of chloride

3.0 Extraction as a Model for Membrane Transport

The classic model for membrane transport that utilizes two immiscible liquids is the bulk liquid membrane,[1-9] or U-tube, experiment, shown below in Figure 3.1. In this scenario the first aqueous phase is likened to the extracellular solution and the second aqueous phase, the so-called receiving phase, is likened to the intracellular solution. The organic solvent separating Aqueous Phase I and Aqueous Phase II is representative of the hydrophobic interior of a lipid bilayer. With the aid of stirring, the diffusion rate of the desired analyte through the organic phase is monitored by the appearance of the analyte in the receiving phase.

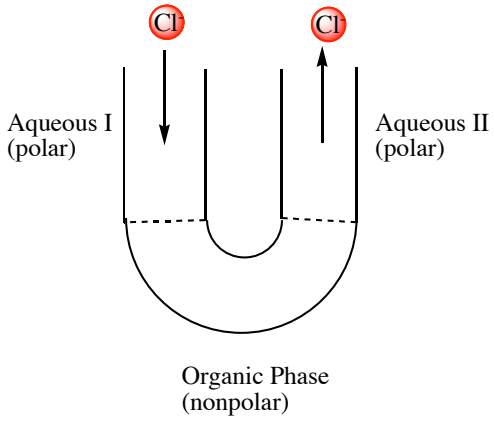


Figure 3.1 Bulk liquid membrane setup. Dichloromethane or chloroform are commonly used as the organic phase ("membrane interior") because of their densities. The process is facilitated by the addition of a stir bar to the bottom of the U-tube and mixing the organic phase gently.

Needless to say, quantitative data from these experiments do not directly correlate to transport rates across true membranes. Nonetheless, qualitative comparisons between

a set of experimental carriers can usually be drawn from such data. Direct transport measurements using live cells, liposome models, or black liquid membranes have become increasingly common in the recent literature. Still, the use of immiscible liquids is not entirely obsolete. Such experiments provide a useful screen to eliminate unsuitable would-be carriers. Further, the combination of extraction and transport data provides a more complete picture of carrier-ion behavior. It was with such intentions in mind that liquid-liquid partitioning studies were carried out in conjunction with transport experiments using liposome model membranes.

3.1 CHLORIDE AS AN ANALYTE

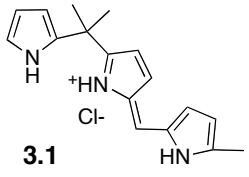
Unlike sulfate, chloride is a spherical, singly charged anion. The ion is moderately sized, being neither so large nor as charge-disperse as sulfate. Being a smaller, harder anion, chloride presents an easier binding target, though a more difficult extraction target. Furthermore, in biology, chloride is one of the most prevalent anions. Phosphate exists in higher concentrations than chloride and presents the highest degree of competition for potential chloride-binding agents. Phosphate, however, is a tetrahedral, charge-diffuse anion. It thus bears little in the way of structural similarity to chloride. Carriers that successfully bind and mobilize chloride do not necessarily exhibit a loss of efficiency in the presence of phosphate. This is because the active binding sites, if made sufficiently selective for chloride, will not generally accommodate phosphate or other larger, nonspherical anions. Fortunately, several ions that would be most likely to present strong competition for binding, including the larger anions bromide and iodide, as well as the smaller species, fluoride, are simply not present to any high degree in biological systems.

3.2 CHLORIDE EXTRACTION STUDIES

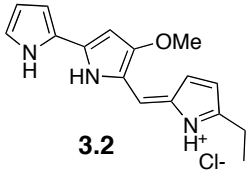
All of the potential extraction agents were examined using the same protocols used for the sulfate partitioning studies. Briefly, a 10 mM solution of the carrier in chloroform was prepared. Inasmuch as there is no A336 present *in vivo* to facilitate anion transport, which was the phenomenon that we were attempting to model with these studies, this co-carrier was not added to any of the solutions being subject to investigation. The final concentrations for each 450 μL carrier solution contained in 1 mL centrifuge tubes were 10 mM, 5 mM, 2.2 mM, 1.1 mM, 0.6 mM and 0.2 mM. In the radiation hood, 450 μL of a spiked sodium chloride or HCl solution were added to each centrifuge tube. The phases were contacted for one hour at 25°C on a rotating wheel to ensure that equilibrium had been reached. The tubes were then removed from the wheel and returned to the radiation hood for subsampling. The phases were separated in the radiation hood. 50 μL of the aqueous phase were subsampled and added to 5.0 mL Ultimax Gold scintillation cocktail in scintillation vials. 250 μL of the organic phase were subsampled and added to 5.0 mL Ultimax Gold scintillation cocktail in scintillation vials. The vials were placed darkness for 30 minutes to allow any photons of light remaining in the samples to fade. The samples were then placed on the beta counter to be read in turn. The raw data counts were corrected for the subsample volume difference between the aqueous and organic phases by multiplying the aqueous counts by five. The data were further corrected for background radiation counts. Distribution ratios were determined by dividing the corrected organic counts by the corrected aqueous counts. All mathematical operations were performed using the software program Excel.

3.2.1 Molecules under investigation

Several molecules produced by other members of the Sessler group were selected for examination as potential anion carriers. Most of the linear molecules examined possessed imine-like nitrogen atoms and were readily protonated. Molecules **3.1**, **3.2**, and **3.3** were isolable as free-base molecules or as HCl salts. Compound **3.2**, prepared by Mr. Apolonio Aguilar, possessed the natural prodigiosin scaffold. All other scaffolds were the products of rational synthesis. Molecule **3.4**, a sapphyrin, represents a macrocyclic porphyrin analogue that, like prodigiosin, possesses an imine-like nitrogen atom and exists in its monoprotonated form at biological pH. Compounds **3.5**, and **3.6** did not possess imine-like nitrogens and were not readily protonated. These compounds were examined for comparison with sapphyrin. Likewise, compound **3.7** was selected for comparison with **3.1**, **3.2**, and **3.3**. A unique feature to **3.7** was the long alkyl chains that were expected to render the molecule more lipophilic.



3.1



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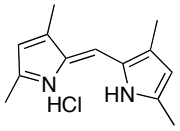

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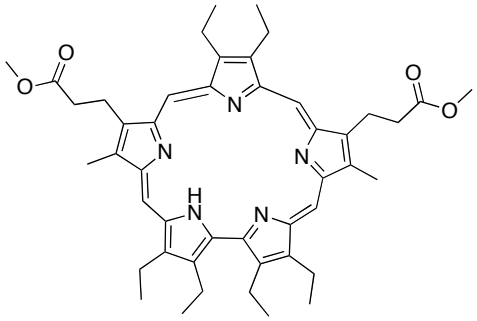

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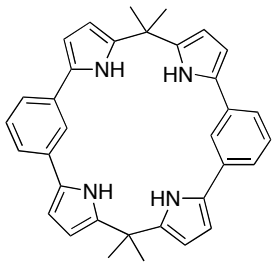

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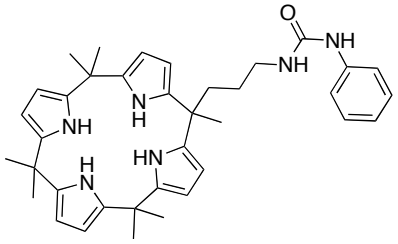
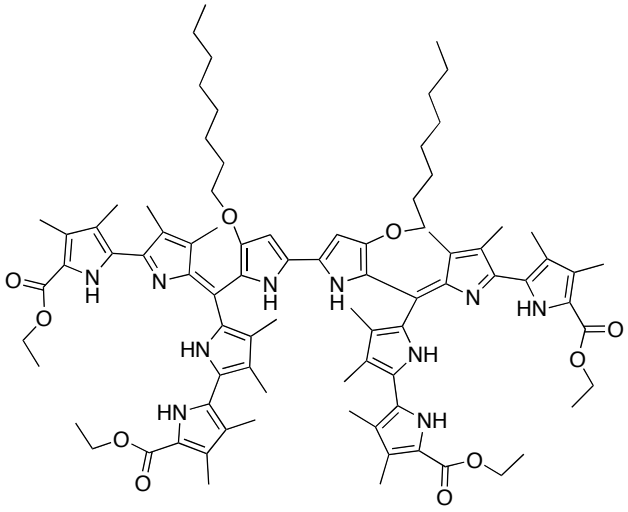


3.6



3.7

3.2.2 pH PROFILE FOR EXTRACTION STUDIES

Carrier **3.1**, a synthetic derivative of prodigiosin, was selected to investigate the effect of pH on extractability. The synthetic ease and stability of this molecule led to its selection for these initial studies. The pH values of 1 mM HCl solutions were adjusted using minimal 0.1 M Tris-Cl such that the change in chloride concentration was negligible. These resulting solutions were spiked with radiotracer and used as the aqueous phases for partitioning experiments at each pH. The pH range examined was 4.5-8.0. Figure 3.2 shows the results of the pH study for Carrier **3.1**. At pH 6.65

extraction was higher than extraction at all other pH values except pH 4.5 at low concentrations of carrier. Extraction at pH 6.65 remained high at higher concentrations of carrier, while extraction efficiency decreased with increasing concentration at pH 4.5. Therefore, pH 6.65 was selected to be the standard pH at which all of the carriers would be tested. At 5 mM carrier concentration, the distribution ratio was found to be essentially unity at every pH examined. In the case of carrier **3.1**, at equilibrium, chloride ions were present at equal concentrations in both the aqueous and the organic phases. Inasmuch as these data were used only to determine the optimal pH at which to conduct the chloride partitioning experiments, they are presented uncorrected for quenching. Here the term quenching refers to the fact that some species absorb or scatter the light emitted from a beta decay event; this light emission is how the instrument detects the presence of radioactive species. Colored species can absorb light and therefore exhibit quenching effects, which would artificially lower the detected concentration of chloride in the either the aqueous or organic phase, resulting in an incorrect distribution ratio. When quenching affects the organic measurement more than the aqueous measurement, the true distribution ratio is higher than that which was calculated from the experimental data. When quenching affects the aqueous measurement more than the organic measurement, the true distribution ratio is lower than that which was calculated from the experimental data.

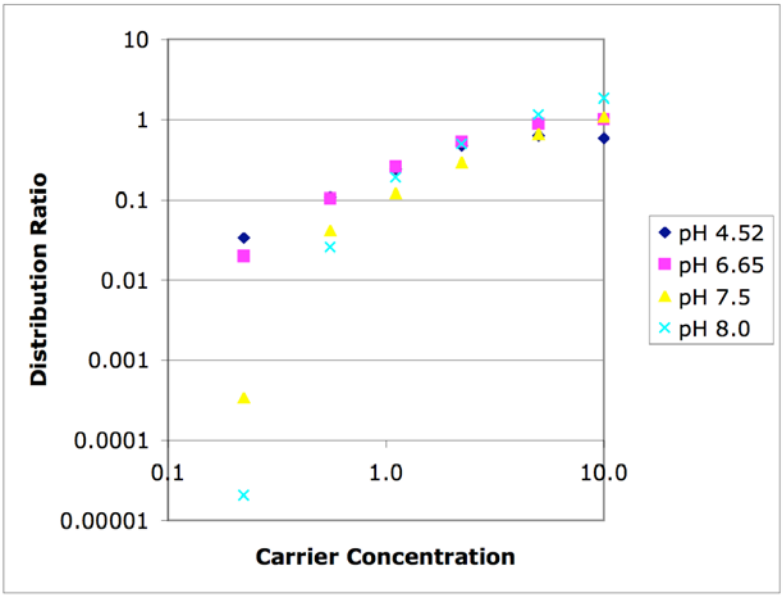


Figure 3.2 Results of pH study using Carrier 3.1. At pH 6.65 the distribution ratios best fit a logarithmic scale of the data. Using 5 mM solutions of Carrier **3.1**, D = 1 at every pH examined.

3.2.3 Chloride extraction efficiency of Carrier 3.1

Of the linear molecules examined, the highest overall distribution ratios were obtained using Carrier **3.1**. Actual extraction data for Carrier **3.1** at pH 6.65 are summarized in Figure 3.3. Quenching effects were not seen to any significant degree. From the equation of the resulting trendline, when fit to a power scale, the exponent is a measure of the association ratio. This ratio was found to be 1:1(carrier molecule to chloride anion). A 1:1 ratio for the complex does not violate electroneutrality and was the expected association for the carrier/ion complex. Higher order complexes would carry a formal charge and would not be expected to partition into the organic solution.

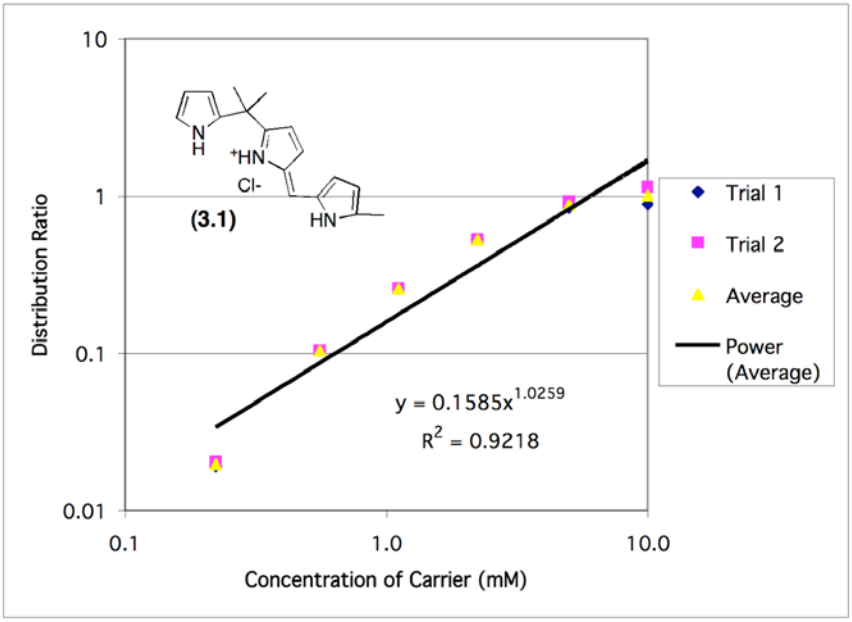
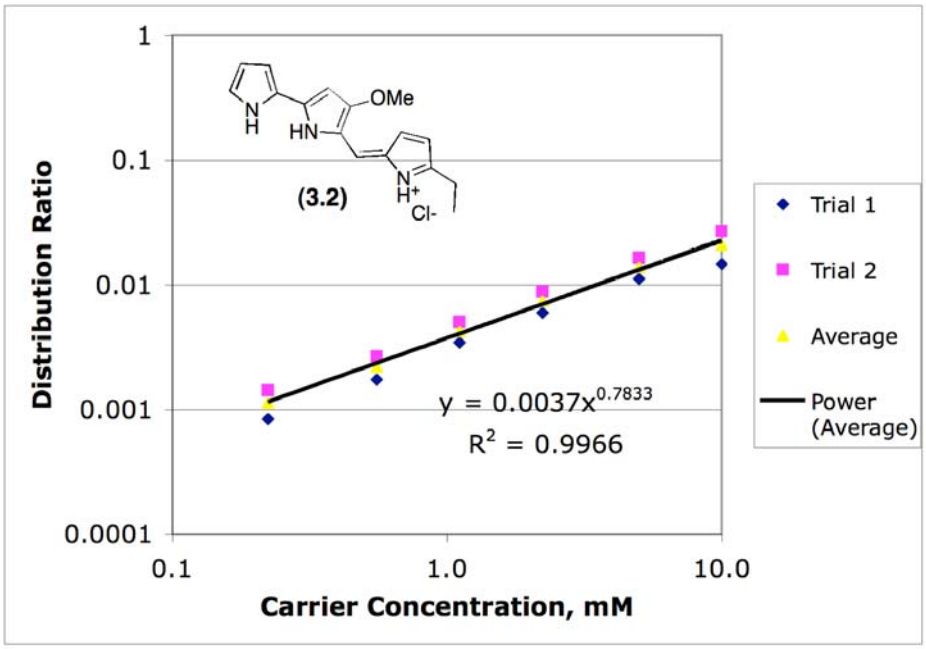


Figure 3.3 Distribution ratios for Carrier 3.1 as determined from data recorded at pH 6.65.

3.2.4 Chloride extraction efficiency of Carrier 3.2

Carrier **3.2** displayed the best curve fitting of the carriers tested, both uncorrected (Figure 3.4a) and corrected (Figure 3.4b) for quenching. The corrected data show slightly lower distribution ratios at all concentrations, indicating that the greatest quenching effects were seen in the aqueous phase. This finding is best rationalized in terms of some amount of Carrier **3.2** partitioning into the aqueous phase. A measure of carrier partitioning was made with the associated findings being presented later in this dissertation. At the present juncture, the most important "take home lesson" is that with a slope exponent value of 0.8228, the corrected data most closely correlate to the formation of a 1:1 complex.

(a)


1
0.1
0.01
0.001
0.0001
Distribution Ratio
0.1
1.0
10.0
Carrier Concentration, mM
OMe
HN
N
H
(3.2)
N
H+
Cl-
y = 0.0037x^0.7833
R2 = 0.9966
Trial 1
Trial 2
Average
Power (Average)

(b)

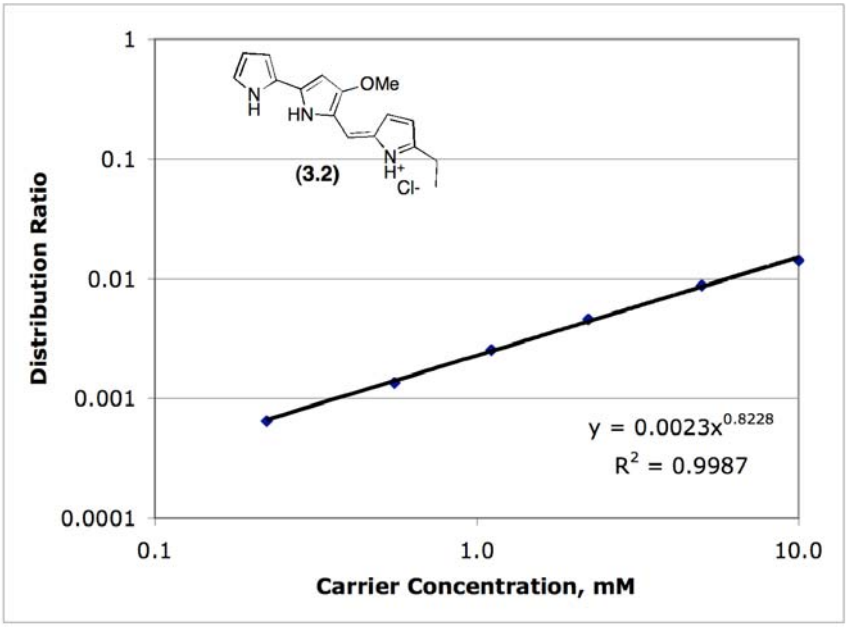


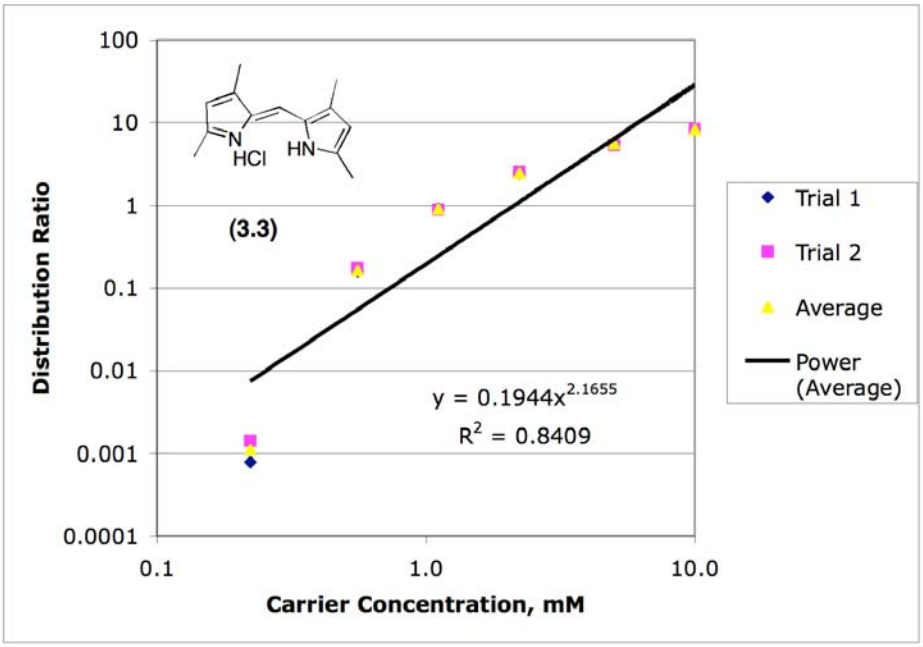
Figure 3.4 Distribution ratios for Carrier 3.2 at pH 6.65. (a) Data uncorrected for quenching. The slight difference is distribution ratios for Trials 1 and 2 is believed to be due to solvent evaporation in one set of vials. (b) Data corrected for quenching. The averaged results from both trials shown in Figure 3.4a are shown as corrected. The trendline and resulting line equation were determined using the software program Excel. As shown in the graph legend, the data trend fit a power equation.

3.2.5 Chloride extraction efficiency of Carrier 3.3

Carrier **3.3** displayed the highest chloride distribution ratio using a 10 mM carrier solution for any of the linear pyrrolic compounds under investigation (Figure 3.5). Additionally, Carrier 3.3 was shown to be significantly more efficient than the natural prodigiosin at all but the lowest concentration tested. The slope equation for the curve-fit trendline indicates 2:1 (or higher order) complex formation of carrier to anion. Unfortunately, the R^2 value is not close enough to unity to allow for a high degree of

confidence in this conclusion. Regardless of the stoichiometry of the extracted complex, however, the key point is that Carrier **3.3** appears to be an efficient extraction agent for chloride anion. Once again, the lowering of the distribution ratios after correction for quenching is indicative of greater quenching effects occurring in the aqueous phase, and therefore some contamination of the aqueous phase by the extraction agent.

(a)



(b)

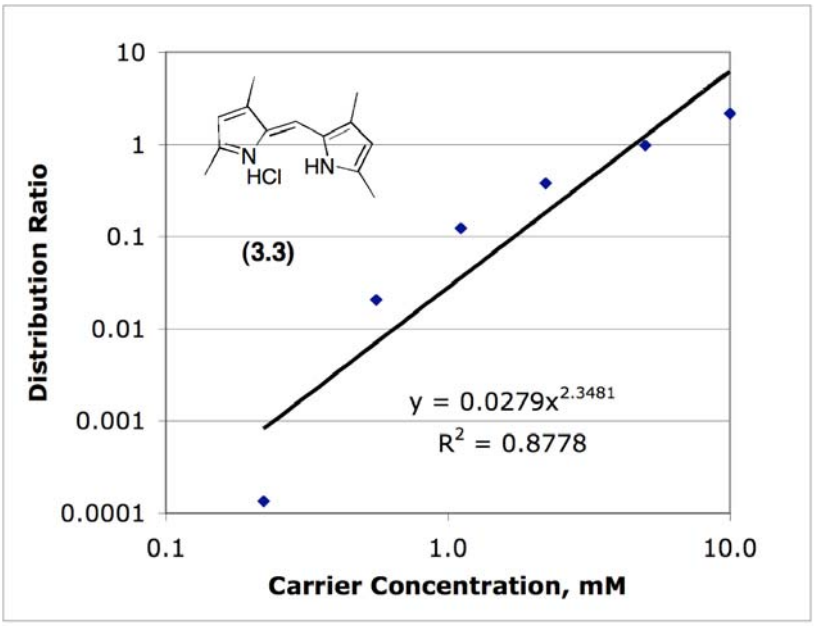


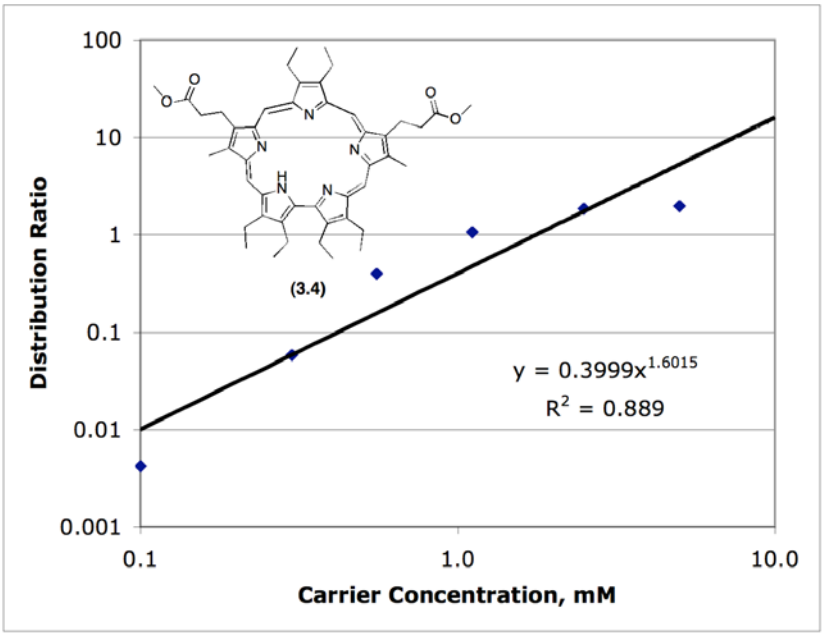
Figure 3.5 Distribution ratios for Carrier 3.3 at pH 6.65. (a) Data uncorrected for quenching. (b) Data corrected for quenching. The averaged results from both trials shown in Figure 3.5a are shown here as corrected. The trendline and resulting line equation were determined using the software program Excel. As shown in the graph legend, the data trend fit a power equation somewhat poorly.

3.2.6 Chloride extraction efficiency of Carrier 3.4

Carrier **3.4** displayed the highest chloride distribution ratios of any of the tested compounds, linear and macrocyclic structures alike. Unfortunately, insufficient material prevented a quench-correction experiment from being performed. Examination of the data presented in Figure 3.6 (a), reveals that the data fit the trendline rather poorly. The two highest concentration data points show little change in their distribution ratios. This was indicative of saturation. Recall that the sapphyrin solutions were prepared at concentrations that were 50% lower than those of the other carriers, while the

concentration of HCl remained identical for all experiments (1 mM); therefore, saturation would be expected to be seen for Carrier **3.4** at lower concentrations than other carriers. A re-examination of the data after removal of the last two data points, as seen in Figure 3.6 (b) shows a better fit of the data to the trendline, R^2=0.9865. The line equation continued to indicate a higher order complex formation, rather than simple a 1:1 association. Regardless of the stoichiometry of the extracted complex, it is clear that Carrier **3.4**, like Carrier **3.3**, is an efficient extraction agent for chloride anion.

(a)



(b)

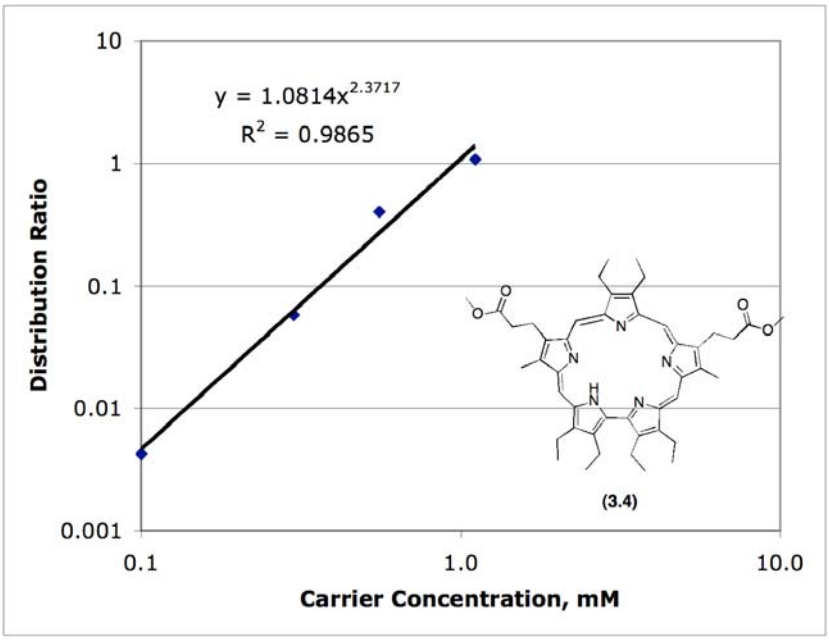


Figure 3.6 Distribution ratios for Carrier 3.4 at pH 6.65. (a) These data are uncorrected for quenching. A lack of material prevented a correction experiment from being performed. (b) A re-fit of the data after elimination of data points at 2.5 and 5.0 mM carrier concentrations. The trendlines and resulting line equations were determined using the software program Excel. As shown in the graph legend, the data trend fit a power equation.

3.2.7 Chloride extraction efficiency of Carrier 3.5

Carrier **3.5** was the first neutral compound to be tested. This hybrid calixpyrrole contains no imine-like nitrogen atoms that can be readily protonated at moderate pH. The data summarized in Figure 3.7 reveal moderate chloride distribution ratios. The data fit the displayed trendline with an R^2 value of 0.8374. Due to the low R^2 value, the assigned 1:2 ratio of carrier to anion should be regarded as tentative. The molecule warrants further investigation of its extraction behavior. Nonetheless, for the purpose of determining potential membrane transport behavior, the data in its current form is

sufficient to support the suggestion that Carrier **3.5** might act as a suitable membrane transport agent.

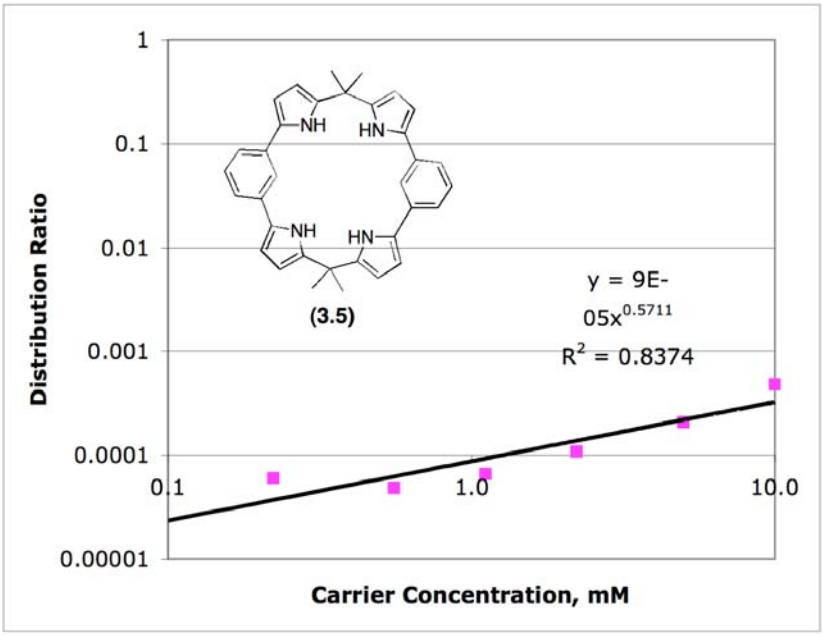


Figure 3.7 Distribution ratios for Carrier 3.5 at pH 6.65. The molecule is not highly colored. Therefore, these data did not require a correction factor due to quenching. The trendline and resulting line equation were determined using the software program Excel. As shown in the graph legend, the data trend fit a power equation somewhat poorly.

There are several aspects to the presented extraction data for **3.5** that justify further investigation of this molecule. The proposed stoichiometry, although not certain, suggests the potential for efficient extraction. In particular, for every molecule of carrier used, two molecules of the desired ion are removed. The size of the cavity indicates that the molecule could accommodate more than one anion. The hydrophobicity of this molecule prevents it from partitioning into the aqueous phase, another favorable characteristic in an extraction agent. Finally, unlike the previous systems examined,

because this molecule is not protonated, any extraction event is an example of a true HCl symport mechanism. It is not the case that a protonated species is simply ion pairing with chloride in organic solution. Rather, a neutral molecule is effectively binding and extracting HCl using only neutral interactions as illustrated with the unappended calixpyrrole shown in Figure 3.8. A counter-ion study would be interesting to address the question of what metal-chloride salts might also be extracted using Carrier **3.5**.

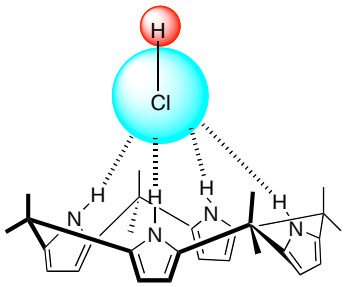


Figure 3.8 An expanded calixpyrrole binding HCl.

3.2.8 Chloride extraction efficiency of Carrier 3.6

Carrier **3.6**, also a neutral species lacking an imine-like nitrogen, demonstrated the most unusual partitioning behavior (Figure 3.9). Seemingly, the molecule extracts chloride more efficiently at very low concentrations as compared to more concentrated solutions. Likely, the urea moiety is bringing about higher-ordered supramolecular associations at higher concentrations, altering the equilibrium of "free" carrier and affectively removing molecules of carrier from available solution. While no reasonable curve-fit could be established from these data, Carrier **3.6** did demonstrate an ability to extract chloride into organic solution and could therefore be construed as a potential

membrane transport agent. This molecule was tested in liposome models. Those results are presented in Chapter 4.

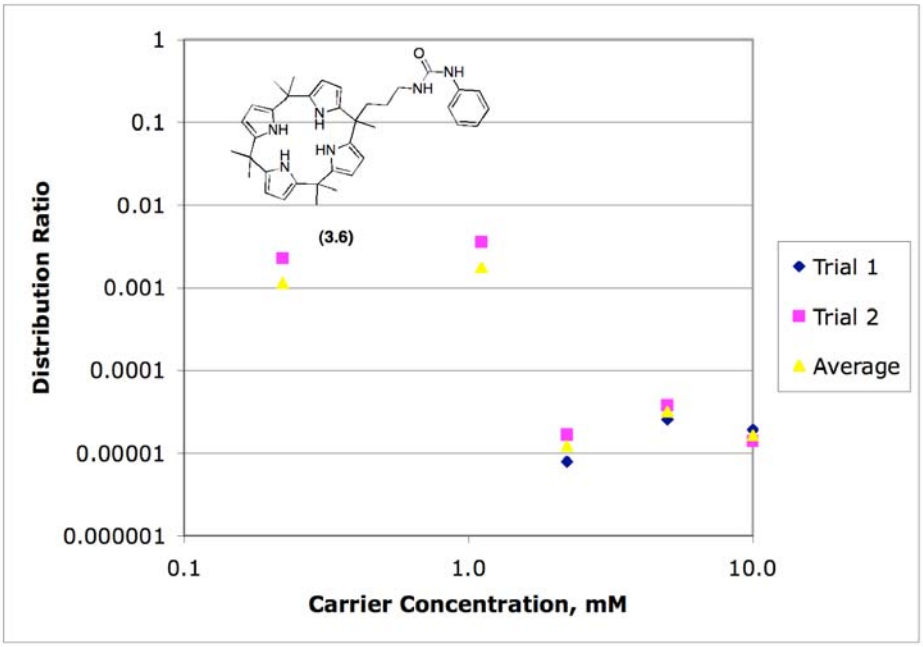


Figure 3.9 Distribution ratios for Carrier 3.6 at pH 6.65. This molecule is not highly colored, therefore no correction factor due to quenching was required. The data do not follow any regular trend.

3.2.9 Chloride extraction efficiency of Carrier 3.7

No chloride anion extraction was seen with Carrier **3.7**. Such an unfavorable result was observed in spite of the high lipophilicity of the system, which would preclude contamination of the aqueous phase. Figure 3.10 shows essentially a line equation of y = 0 for all values of x. Carrier **3.7** does, however, possess two tetrapyrrolic binding pockets that might allow for the extraction of larger anionic species.

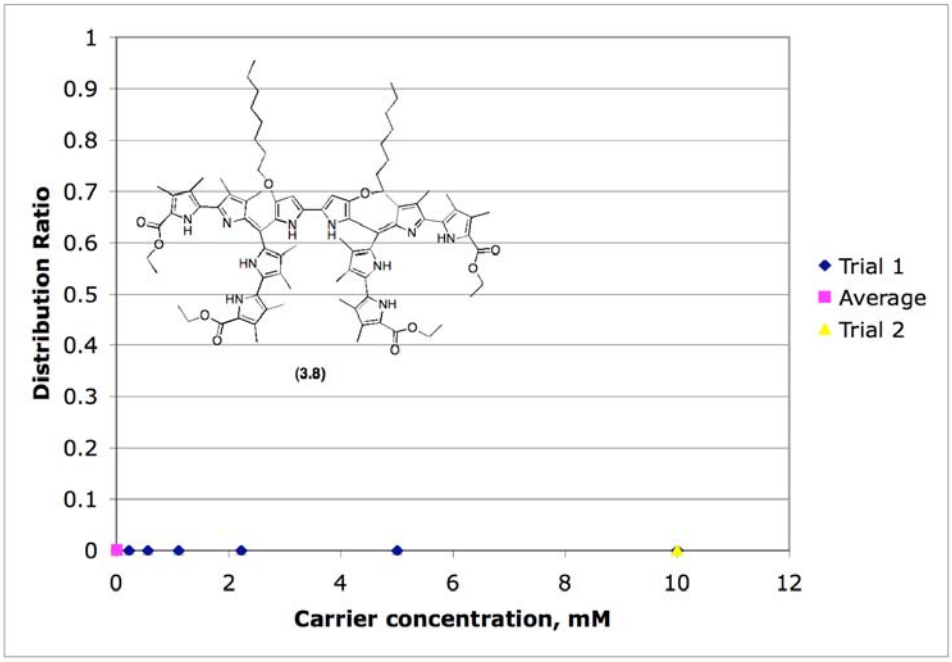


Figure 3.10 Distribution ratios for Carrier 3.7. No extraction of chloride was detected for Carrier **3.7**.

3.2.10 Chloride Extraction Summary

To summarize the chloride extraction studies, Carriers **3.1**, **3.2**, **3.3**, **3.4**, **3.5**, and **3.6** all demonstrated measurable chloride extraction and the potential to function as membrane transport agents as indicated in Figure 3.11. Due to insufficient material, Carrier **3.5**, though interesting, was not subjected to liposome model studies. Carriers **3.1** and **3.2** show good curve-fits that corresponded to the formation of 1:1 carrier : anion complexes, with Carrier **3.1** displaying the highest overall distribution ratios for the linear molecules tested. The data for Carrier **3.3** indicated the formation of a possible 2:1 complex, as well as the highest single distribution ratio among the linear molecules examined, at least at a 10 mM carrier concentration (Figure 3.12). Carrier **3.4** demonstrated the highest distribution ratios obtained in the study. However, a

logarithmic plot of the data was found to deviate from linearity, possibly due to saturation. Examination of the lower concentrations revealed a linear relationship and the possible formation of a higher-ordered complex. Carrier **3.5** demonstrated the lowest distribution ratios of the molecules tested as reflected in Figure 3.10. While the distribution ratios were significantly lower than the ratios obtained for **3.1**-**3.4**, the data are impressive given the inference that HCl is being extracted by a neutral species. Carrier **3.6**, also an unprotonated species, demonstrated highly nonlinear behavior. This is rationalized in terms of the molecules being organized into high-ordered supramolecular structures at higher concentrations. The solution behavior of this species warrants further investigation because, in spite of the complexities, this molecule was shown to extract chloride anion into organic solution.

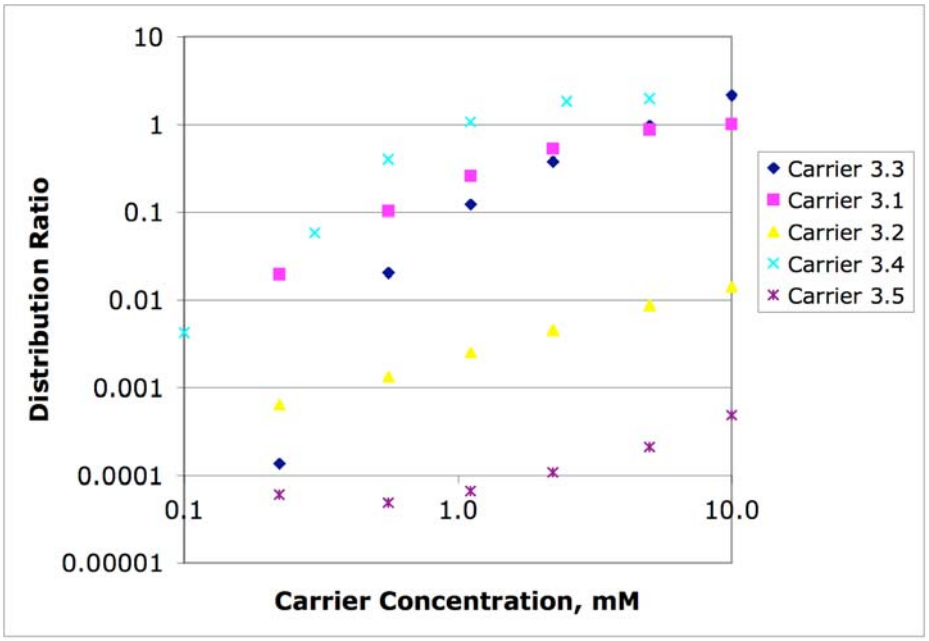


Figure 3.11 Summary of chloride distribution ratios. These carriers all showed successful extraction of chloride into chloroform solution.

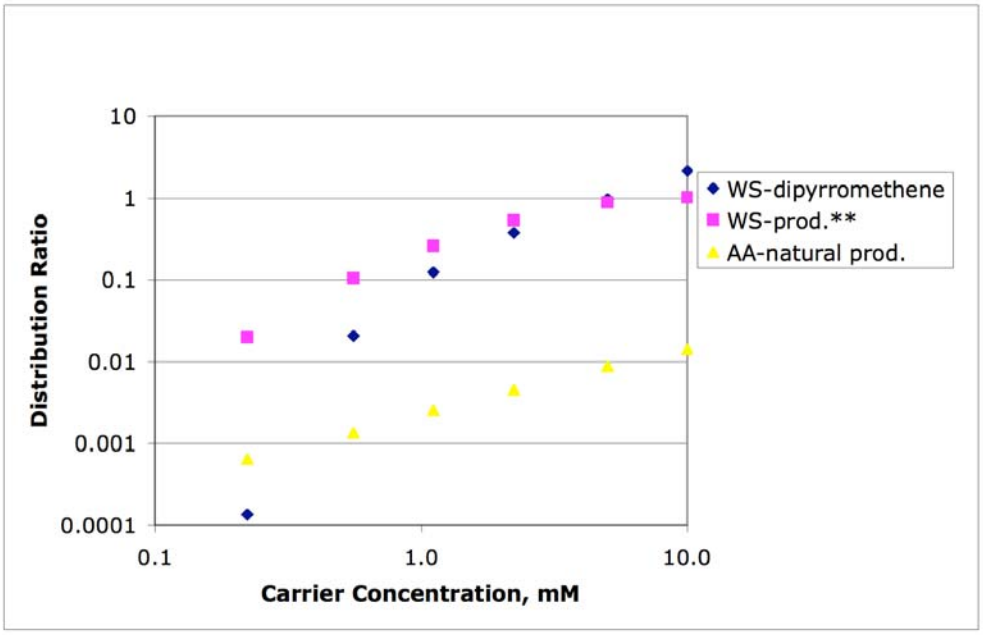


Figure 3.12 Comparison of prodigiosin and experimental derivatives. Both experimental derivatives demonstrated higher chloride extraction efficiencies than the natural prodigiosin.

3.3 CHLORIDE BINDING CONSTANTS

The chloride-binding data discussed below was gathered by other members of the Sessler Group, particularly Won-Seob Cho, and not by the author. These binding constants are, however, relevant to the overall discussion of whether a given receptor system is or is not expected to extract and transport chloride anions. They are thus included for the sake of completeness.

3.3.1 Binding data for Carrier 3.1

Binding data for this molecule is still being determined. Based on the structure of the molecule, the chloride association constant is presumed to be somewhat lower than

that of natural prodigiosin, Carrier **3.2**. Nonetheless, the chloride extraction behavior exhibited by **3.1** was among the highest extraction behavior for any of the molecules examined in this dissertation. These data are summarized in Table 3.1.

3.3.2 Binding data for Carrier 3.2

The chloride binding constant in acetonitrile was found to be 586,300 M^{-1} by Mr. Won-Seob Cho using Isothermal calorimetry (ITC). While the association constant for this carrier was among the highest of the molecules studied, this carrier displayed moderately low chloride extraction behavior. These data are summarized in Table 3.1.

3.3.3 Binding data for Carrier 3.3

The chloride binding constant in acetonitrile was found to be 74,000 M^{-1} by Mr. Won-Seob Cho using Isothermal calorimetry (ITC). Although the association constant was lower than that of Carrier **3.2**, the distribution coefficient was a full order of magnitude higher and the distribution ratio using a 1 mM carrier solution was 2 orders of magnitude higher than the corresponding results for prodigiosin, **3.2** (see Table 3.1).

3.3.4 Binding data for Carrier 3.4

The reported association constants for sapphyrin, Carrier **3.4**, were determined using fluorescence titrations in both methanol (MeOH), ca. 100 M^{-1}, and dichloromethane (DCM), 18,000,000 M^{-1}.[10] In the case of sapphyrin, the molecule exhibited strong binding as well as strong extraction behavior, unlike prodigiosin, Carrier **3.2**, where in spite of strong binding to chloride, efficient extraction was not seen.

3.3.5 Binding data for Carrier 3.5

The reported association constants for this hybrid calixpyrrole were determined in dichloroethane (DCE) using ITC, 5,600,000 M^{-1}, and in dichloromethane (DCM) by NMR titration, >10,000, by Mr. Deqiang An.[11] This hybrid calixpyrrole displayed very low chloride extraction ability despite the high association constant of the molecule for chloride anion (see Table 3.1).

3.3.6 Binding data for Carrier 3.6

The chloride binding constant in acetonitrile was found to be 480,000 M^{-1} by Mr. Won-Seob Cho using Isothermal calorimetry (ITC). The chloride affinity of this urea-appended calixpyrrole was among the highest studied, however, the extraction ability of **3.6** was among the lowest for the set of carriers studied by liquid-liquid extraction.

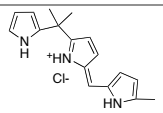
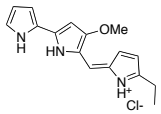
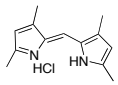
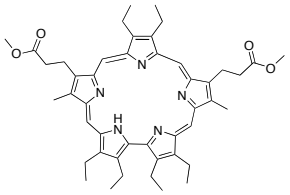
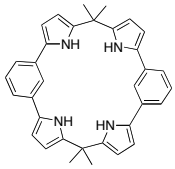
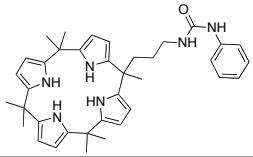
	Carrier	Distribution Ratio Coefficient	Distribution Ratio (1mM) at 300 sec	Association Constant (K_a), M^{-1}
3.1		0.1585	0.26085	N/a
3.2		0.0023	0.00255	5.86×10^5
3.3		0.0279	0.12395	5.80×10^5
3.4		0.3999	1.07319	ca. 100[a], 18,000,000[b]
3.5		0.00009	6.6×10^{-5}	< 10,000[c], 5.60×10^6 [d]
3.6		N/a	0.00178	4.80×10^5

Table 3.1 Chloride binding and extraction correlation summary. All of the binding data were determined in acetonitrile using Isothermal calorimetry (ITC), with the exception of the sapphyrin **3.4**, which was studied in both methanol ([a]) and dichloromethane([b]) using fluorescence titrations and **3.5**, which was studied in dichloromethane([c]) using ITC and in dichloroethane ([d]) using NMR.[10] Aliquots of tetrabutylammonium chloride in acetonitrile were titrated into a solution of host molecule in acetonitrile at 298K. Mr. Won-Seob Cho is kindly thanked for providing these data. Distribution coefficients were determined by the slope of the data trendline and Distribution ratios were compared between molecules at a carrier concentration of 1 mM.

3.3.7 Correlation of anion binding data and extraction behavior

In the case of prodigiosin, the synthetic analogues of prodigiosin, and both calixpyrrole derivatives, an inverse correlation between binding affinity and extraction ability was seen. The native prodigiosin, having the highest association constant, proved less effective for extraction than either calixpyrrole or the synthetic analogues of prodigiosin. The experimental derivative displaying the lowest association constant demonstrated significantly higher chloride extraction ability. Only in the case of sapphyrin was there a linear correlation of anion binding and anion extraction behavior.

3.4 SUMMARY

The association constant data provided to the author by (mostly) Mr. Won-Seob Cho makes it clear that anion affinity per se is not the primary determinant in defining the efficiency of extraction agents. However, sapphyrin, which in its diprotonated form shows a very high affinity for Cl^- in $CHCl_3$, and dipyrromethene demonstrated the highest chloride distribution ratios. On this basis it might have been postulated that sapphyrin and dipyrromethene would display the highest rates of chloride transport. As will be discussed in Chapter 4, this expectation was not met.

3.5 EXPERIMENTAL

Chloride partitioning experiments at oak ridge national laboratory

A 1:10 dilution of an original stock 0.005 N solution of $NaCl^{36}$ in H_2O was used in all chloride extraction experiments as the beta source. The stock solution contained 9.35×10^{-4} Ci in 0.1518 mL as of 06/13/03. 10 mM solutions in chloroform of each carrier were prepared, with the exception of sapphyrin, in which case a 5 mM solution in chloroform was prepared. 1mM HCl solutions were buffered to pH 4.5 and pH 6.5 using 0.1 M Tris-Cl and spiked with 1μL/mL of the Cl^{36} tracer solution (diluted stock) in the radiation hood. To six 1-mL centrifuge tubes were added 10 mM solutions of the carrier in chloroform in the following amounts: 450 μL, 225 μL, 100 μL, 50 μL, 25 μL and 10 μL. An additional volume of chloroform was added to each tube in the following amounts: 0 μL, 225 μL, 350 μL, 400 μL, 425 μL, and 440 μL, respectively. Each centrifuge tube contained 450 μL of the chloroform carrier solution in the following concentrations: 10 mM, 5 mM, 2.2 mM, 1.1 mM, 0.6 mM, 0.2 mM, respectively. In the radiation hood, to each centrifuge tube 450 uL of spiked aqueous chloride solution were added. The centrifuge tubes were sealed, removed from the radiation hood, tested for radiation leaks, and placed into 50-mL conical vials packed with Kimwipes. The conical vials were sealed and placed on a mechanical rotating wheel for one hour. The vials were then removed and the inner tubes were centrifuged for 10 minutes to re-separate the layers. 50 μL of each aqueous layer were subsampled, while 250 μL of each chloroform layer were subsampled. The subsamples were added to 10 mL of scintillation cocktail each and placed into a beta scintillation counter. From the counting data, distribution ratios were calculated using the software program Excel.

3.6 REFERENCES

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Chapter 4: Membrane Transport

4.0 LIPOSOMES

Liposomes have been used extensively in the development of systems for drug delivery[1-4] and the study of membrane-protein interactions.[5-13] The studies performed for this dissertation exploit the use of liposomes as models for anion transport across membranes. A successful experimental model for any complex system requires intimate knowledge of the characteristics of the model. To that end, a discussion of the relevant aspects of liposome biophysics,[14-26] preparation, stability, and characterization is in order.

4.0.1 Liposome biophysics

As was discussed in the introduction to this dissertation, liposomes are spontaneously formed when a dry lipid film is hydrated. Spherical vesicles form that entrap solvent, and any solutes that are present, inside the liposomal interior. Because liposomes are generally composed of only one or two lipids, liposome membranes are not as efficiently packed as living biomembranes. They are less stable and subject to more leakage. However, there are many advantages to utilizing liposomes. Because one can select the lipid components and molar ratios, many features of the resulting membrane, including fluidity and surface charge, can be finely tuned to the needs of the researcher.

Interactions at the liposome surface to some extent dictate transport events.[14] Electrostatic attractions or repulsions between the charged headgroups and the approaching solute (anionic in our studies) will enhance or deter transport. Using surface techniques such as Second Harmonic Generation (SHG), researchers can detect

interactions of only those molecules adsorbed to the liposome surface, ignoring those molecules in bulk solution.

Lipid composition has a strong effect on transport events. Fluidity of the bilayer is one control mechanism for the influx and efflux of materials from cells. The ability of some lipids to adopt conformations other than the lamellar (L-α) phase in response to certain stimuli serves to change the fluidity of the membrane, creating microbends and altering the curvature of the bilayer surface. Phosphatidylserine has been implicated by some researchers as being particularly responsible for these bending events.[18,19.] In any event, the underlying membrane structure/function studies have been useful in a range of research areas running the gamut from basic biology[17] to drug delivery.[23] In addition to physical studies, a number of simulation studies have been performed to understand further the complexities of lipid/membrane biophysics.[27-29.]

4.0.2 Preparation of liposomes

Liposomes have been prepared in a wide range of sizes, extending from small, unilamellar vesicles (SUVs) on the order of 20 nm to giant unilamellar vesicles (GUVs) on the order of 20 μm.[30] By sonicating a crude batch of liposomes at various temperatures, SUVs of uniform and reproducible sizes can be formed.[31] The entrapped volume of SUVs are generally too small to allow for their use inn transport experiments. Therefore, large unilamellar vesicles (LUVs), on the order of 100-600 nm in diameter, are most commonly used for these kinds of studies. While sonication may be used for preparing SUVs, it fails to provide uniform LUVs. The process for creating uniform LUV suspensions from crude multilamellar vesicles is extrusion.[21,32-36] In this process, the crude MLV suspension is repeatedly forced through a polycarbonate membrane until homogeneity is obtained. By selecting the desired membrane pore size, complete control

over the resulting LUV suspension is maintained. Care must be taken that the LUV suspensions do no drop below the transition temperatures for the phospholipids. Otherwise, the liposomes will undergo a phase transition from liquid crystalline to gel, with a resulting 25-30% reduction in the entrapped volume.[21]

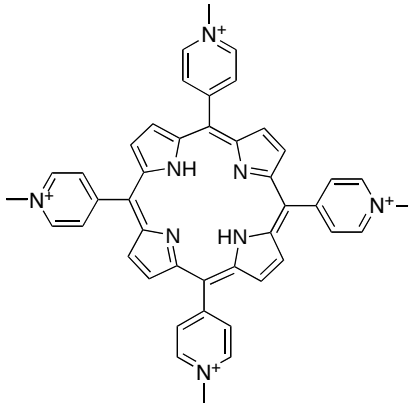
Once formed, a number of assays have been developed to determine the final lipid composition[37] and liposome characteristics after preparation.[38-40] To be useful in transport studies, the structural integrity of the liposome bilayer in both the presence and absence of a carrier must be ascertained. Should the liposome bilayer degrade, the detection of entrapped particles that have leaked through holes in the membrane could be falsely ascribed to transport via a carrier. Thus, leakage experiments are performed as a control measure.[5, 41-43.] Generally, a fluorescent dye is incorporated into the vesicle interior. While entrapped, the inherent fluorescence is severely hindered. Upon addition of carrier species, the fluorescence intensity of the dye compound is monitored over time. Any increase in fluorescence is due to the dye leaking across the membrane. The implication of such a result is that the potential carrier does not successfully transport selected materials across the bilayer; rather, the potential carrier degrades the membrane, allowing any and all entrapped materials, including the dye, to escape.

The effective entrapment of particles in a vesicle interior is key to the success of liposome-based transport studies. Simple hydration of a lipid film with a solution of the desired species does not ensure an entrapment that is both uniform and efficient. Post-hydration procedures have been developed that increase the concentrations of entrapped particles within the interiors of vesicles. By repeatedly subjecting the liposome suspension to warm water and liquid nitrogen baths, the entrapment efficiency can be improved by an order of magnitude or greater.[44-45] Such freeze-thaw cycles are commonplace in any modern liposome preparation protocol. Recent literature references

recommend the use of at least five cycles to obtain a viable preparation. For the studies conducted in this dissertation, nine freeze-thaws were used.

4.1 PREVIOUS WORKS

In recent years, liposome models have become very popular for a range of disciplines. An attempt to provide an overview of all aspects of current work involving liposomes would be futile. Nonetheless, in order to impart an appreciation for our own efforts in this area, some previous work, particularly in relation to anion transport, is presented here. The association of liposomes with organic molecules such as quinine,[46-48] 6-fluoroquinolone,[49] dipyridamole (a coronary vasodilator),[50] and grepafoxacin (an antimicrobial agent)[51] have received a good deal of attention. Hunter and co-workers have examined cooperative binding effects at liposomal surfaces using synthetic receptors that have been incorporated into membranes.[52] Of more interest to the Sessler group, the incorporation of water-soluble porphyrins[53-55] (such **4.1** shown below) into liposomes have been examined kinetically.[56]

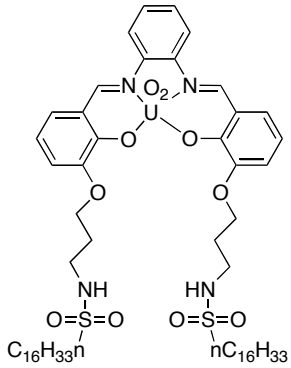


4.1

The passage of small molecules, neutral and ionic, have been studied by theoretical[57-59] and experimental[60-77] means.

4.1.1 Transport of anionic materials

Reinhoudt and coworkers have developed neutral uranyl-based carriers, **4.2**, that transport salts across membranes.[72] Lipophilic salts such as $NaBPh_4$, NBu_4NO_3, PPh_4Cl, and similar salts were successfully carried across membranes.



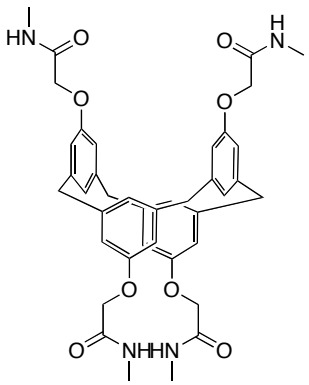
4.2

4.1.2 Transport of chloride and HCl

Lipophilic salts such as those used by Reinhoudt are not found in biological systems. Transport agents capable of mobilizing more common, biologically relevant salts (NaCl, KCl, etc.) are more attractive targets for potential drug design. A recent molecular dynamics simulation study examined the effects of anion charge density on lipid bilayers. The results indicated that the anions which affected membrane disruptions paralleled the Hofmeister series in both order and magnitude. The fallout of this study reinforced the energetic difficulties inherent to efficient chloride transport. Chloride was

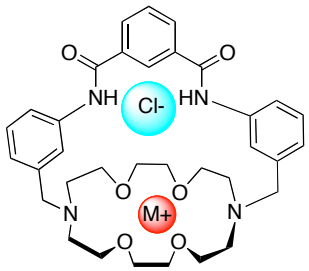
shown to be sufficiently charge-dense that it did not penetrate the interior of the bilayer to an appreciable extent, unlike larger anions such as perchlorate.[59]

Channel-forming molecules based on calixarenes[71,74] and crown ethers[75] were prepared and were shown to facilitate the movement of chloride across a liposome membrane. Calixarenes such as **4.3** have been shown to facilitate H^+/Cl^- symport through a channel.



4.3

In addition to channel-forming molecules, the Smith group at Notre Dame has developed ditopic receptors, such as **4.4**, which promote M^+/Cl^- symport as well.[73]

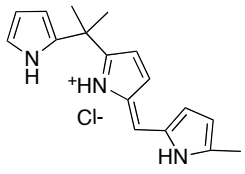


4.4

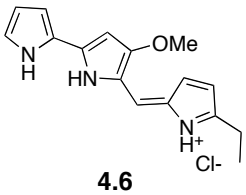
Unlike the crown compounds under investigation in the Smith group, the molecules examined in this dissertation are all pyrrole-based, in keeping with the intention to investigate structural aspects of native prodigiosins that relate to its putative ability to act as an anion carrier in its protonated form.

4.2 MOLECULES STUDIED

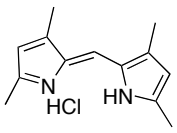
The majority of the molecules used in the transport studies presented below were previously used in the liquid-liquid partitioning experiments presented earlier. The exceptions are **4.8**, which was developed subsequent to the experiments carried out at ORNL, **4.11**, which was substituted for **4.10** due to solubility problems, and **4.12**, which was unavailable during the time the partitioning experiments were being conducted. Carrier **4.10**, a sapphyrin, proved to be sufficiently insoluble in aqueous media, that a microliter quantities of a 1.0 mM solution in DMSO immediately crashed out as a solid upon addition to the liposome suspensions. Carriers **4.9** and **4.12** were the only molecules lacking imine-like nitrogens used in these studies. As previously discussed, any transport demonstrated by **4.9** and **4.12** was expected to be brought about by hydrogen-bonding interactions alone, without the aid of ion-paring.



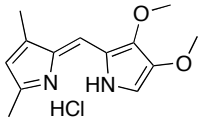
4.5



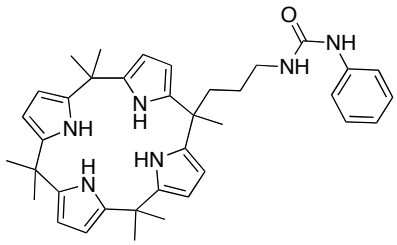
4.6



4.7



4.8



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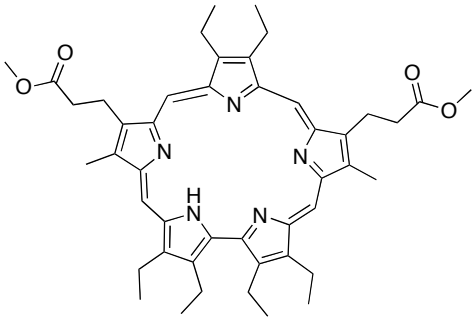
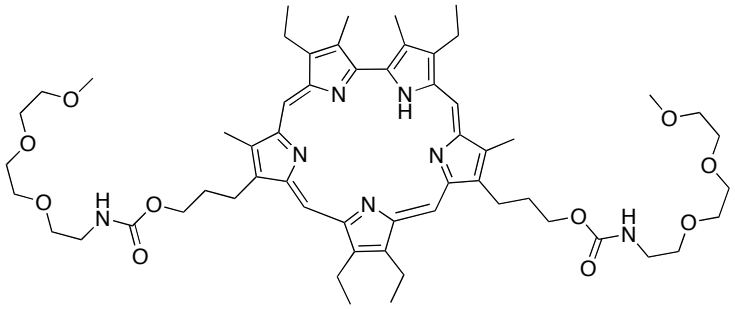
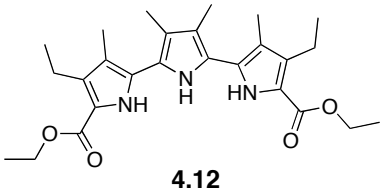


4.10

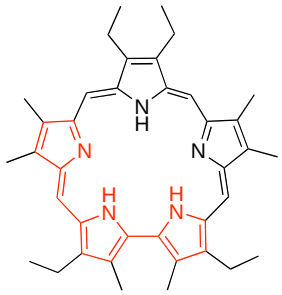


4.11



4.12

Carrier **4.6** was chosen for study as a control compound because prodigiosin has previously been implicated in H^+/Cl^-symport in living cells. Carrier **4.5** was chosen for comparison with Carrier **4.6** because it possesses a similar tripyrrolic structure, but lacks either a bipyrrole moiety or complete conjugation. Carriers **4.7** and **4.8** both possess the dipyrromethene unit of prodigiosin. The study of these two compounds allows for an investigation into the relative importance of methoxy substitution on the anion transport ability of a carrier species. The sapphyrins, Carriers **4.10** and **4.11**, are essentially macrocyclic versions of prodigiosin. By studying these two compounds, it is possible to explore the potential advantages of additional hydrogen bond donors and stronger anion binding.



Carriers 4.9 and 4.12 were chosen to examine to transport abilities of molecules that do no readily protonate over a biologically relevant pH range. In these cases, one cyclic and one linear, respectively, binding and transport would occur solely through neutral interactions. With this set of molecules, a number of potential factors in anion transport ability are considered, i.e. macrocyclic effects, neutral vs. charged binding interactions, stronger vs. weaker binding interactions and size.

4.3 RESULTS OF CHLORIDE INFLUX STUDIES

Chloride influx studies proved to be an undesirable means of analyzing these carriers. The entrapped volume : total volume ratio is less than 30% for any liposomes of the size that could be prepared and characterized readily. At best, observable changes were statistically unreliable. Nonetheless, brief descriptions of the experiments and the results are presented here for the sake of completeness.

4.3.1 Fluorescence studies using MQAE

In these experiments, 2 mM N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE)[78], a chloride-selective fluorescent dye, was incorporated into the liposomes by hydrating the dried lipid films with a solution of the dye in water. A sodium chloride solution was added to the liposome suspension in a fluorescence cuvette, followed by addition of the carrier compound. Rather than quenching in the presence of chloride, introduced into the interior of the liposome via the carrier, the fluorescence of the dye increased over time. A control titration of dye and carrier indicated that a fluorescent excimer was being formed, the intensity of which masked any quenching due to the presence of chloride. The experiment demonstrated the ability of the carriers to cross the membrane, but did not successfully demonstrate chloride transport.

4.3.2 Visual study using $AgNO_3$

Due to this excimer formation, fluorescent studies were abandoned in favor of electrochemical analytical techniques. Prior to commencing these studies, a brief proof of principle experiment was carried out using silver nitrate in an effort to demonstrate that chloride was entering the liposome. Towards this end liposomes were prepared

containing $AgNO_3$ encapsulated inside. Upon addition of sufficient carrier and NaCl to the external solution, some slight cloudiness was seen. The change in light transmittance was attributed to the intra-liposomal formation of AgCl, which is insoluble. This experiment provided a qualitative indication that chloride was entering the liposome. This was ascribed to the experimental carrier, since no decrease in light transmittance was seen when sodium chloride was added to the solution *sans* carrier. Quantitative data were unobtainable with this method. Ultimately, influx experiments were abandoned in favor of the more statistically-reliable efflux measurements.

4.4 RESULTS OF CHLORIDE EFFLUX STUDIES

4.4.1 Liposome considerations

Phosphatidylcholine and phosphatidylserine in an 8:2 molar ratio was chosen as the lipid recipe for the liposomes used in these studies. The addition of cholesterol did not improve the stability of the liposomes and resulted in irreproducible transport data. Phosphatidylcholine is a neutral species and phosphatidylserine is an anionic species. The resulting liposomes carried a small net negative charge on their surfaces. This surface charge was not sufficient to hinder anion transport by repelling chloride anions from the membrane surface. Initially, other components and ratios were tried in an attempt to obtain useful liposome preparations. However, none proved superior to those obtained from 8 : 2 phosphatidylcholine : phosphatidylserine.

Various sizes of liposomes were examined. Liposomes having 100 nm, 200 nm, 400 nm, 600 nm, and 800 nm diameters were all prepared and tested against several of the carrier compounds for transport behavior. However, only the experiments using the 200 nm vesicles yielded consistent and reproducible results. It is believed that in the case

of the 100 nm vesicles, the ratio of entrapped volume to the total solution volume was too low to demonstrate statistically relevant and consistent data. Calculations using the molar volume of phosphatidylcholine and the mean diameter estimated the maximum trapped volume to be 7.6% of the total volume. Literature estimates place entrapped volume to total solution volume ratio to be less than 1% for a suspension of 100 nm liposomes prepared in the same concentration as those used in the presented work.[45] On the other hand, in the case of the larger vesicles, there is a greater chance that MUVs were still present after extrusion and that the liposome suspensions were not uniform and thus not suitable for reproducible experiments.

4.4.2 Lazar Microelectrode

Experiments were attempted using a chloride-selective microelectrode designed by Lazar Research, Inc. The response time of the electrode proved to be too slow to observe transport events at any concentration of chloride, liposome diameter, or suspension volume attempted. Further, the electrode was replaced five times because of excessive leakage. None of the replacements demonstrated an improvement in function. A new Accumet brand chloride-selective electrode was thus purchased from Fisher Scientific.

4.4.3 Accumet Electrode

The Accumet electrode proved invaluable to these studies. The response time and sensitivity of this electrode allowed experiments to be conducted in which data points were taken every ten seconds. Calibrations were performed manually prior to each set of transport experiments using 100 mL standard solutions of sodium chloride (1 ppm, 10

ppm, 100 ppm, 1000 ppm, and 10 000 ppm), with 2 mL 5 M $NaNO_3$ added as an ionic strength adjuster (ISA). A typical calibration curve is presented in Figure 4.1.

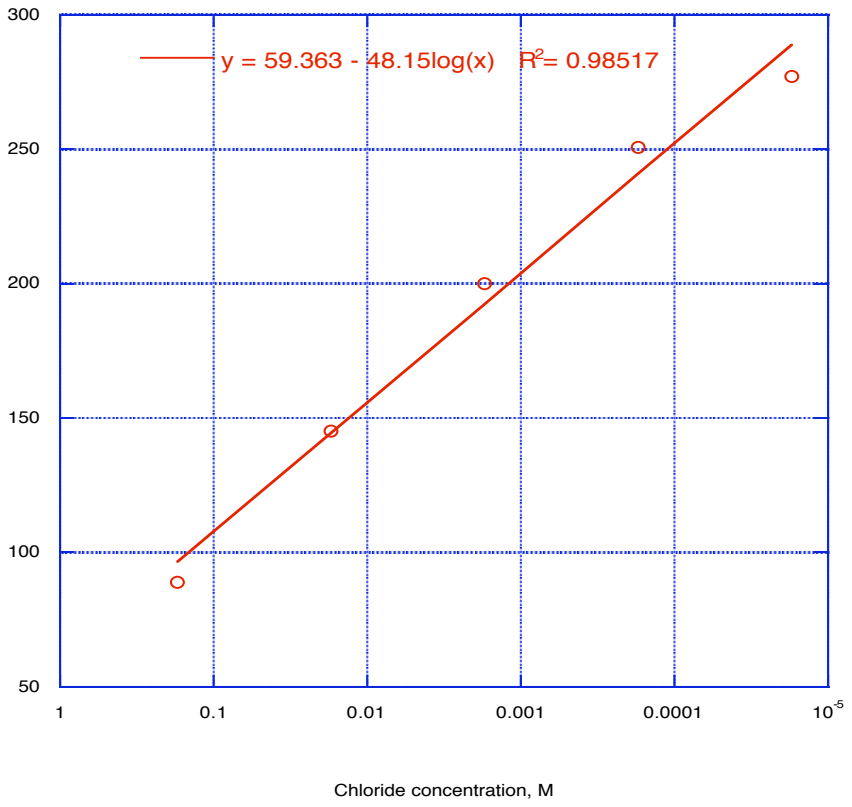


Figure 4.1 Chloride calibration curve. A typical calibration for the Accumet chloride-selective electrode. Fresh calibrations were performed after one hour to compensate electrode drift.

The experimental protocol was maintained consistently for each carrier examined. Liposomes containing 500 mM NaCl were suspended in a buffer solution containing 500

mM $NaNO_3$ and 5 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) (adjusted to pH 7.4) or 5 mM 2-(N-morpholino)ethanesulfonic acid (MES) (adjusted to pH 5.5). The majority of the experimental carrier systems were studied at both pH values. Natural prodigiosin, **4.6**, displayed such high transport rates at pH 7.4 that no studies were conducted at a lower pH. The terpyrrole, **4.12**, demonstrated essentially no transport at pH 7.4, and was abandoned in favor of the other carriers. After calibration, the electrode was allowed to stabilize for ten minutes in the liposome/buffer suspension. Aliquots of a 1 mM carrier solution in dimethylsulfoxide (DMSO) were introduced via microsyringe (0.5 – 4.0μL). The electrode response was monitored over a period of five minutes. The reading at t = 0 seconds, prior to addition of the carrier, was taken as 0% chloride release. Readings were taken every ten seconds for the first minute and every thirty seconds for four additional minutes. At t = 5.0 minutes, a bolus shot of 20 μL of a 100 mM solution of octaethylene glycol monododecyl ether was added via syringe to lyse the membranes. Readings were taken every thirty seconds for an additional two minutes. The final reading at t = 7.0 minutes was taken at 100% chloride release from the vesicles. The data were normalized against the defined 0% and 100% chloride released measurements using the software program Kaleidagraph such that each mV reading was converted to a percentage of total chloride release from the liposomal interior.

4.4.3.1 Chloride transport rates with Carrier 4.5

The tripyrrolic derivative of prodigiosin, Carrier **4.5**, displayed modest transport at both pH 7.4 and ph 5.5 (Figure 4.2 and Figure 4.3). Slightly higher transport rates were seen at pH 7.4 and at both pH values tested the percent of chloride release varied in

a linear fashion with time at every concentration of carrier. The maximal chloride efflux obtained at either pH was approximately 20%.

There are two potential transport mechanisms by which the carrier is facilitating the efflux of chloride from the interior of the vesicles. Experiments to determine the transport mechanism for each carrier are addressed later in this chapter. An antiport mechanism (Scheme 4.1), in which a counter anion such as nitrate from the extra-liposomal solution is carried across the membrane and exchanged for chloride is considered as being most probable. There is literature precedent for such a mechanism, wherein chloride efflux rates were found to drop off significantly when nitrate was replaced with sulfate or perchlorate in the extra-liposomal solution.[73] Under the conditions of the experiment, a symport mechanism (Scheme 4.2) is also possible. In this case, HCl would be the species removed from the liposome interior and the result would be a net pH gradient across the liposomal barrier.

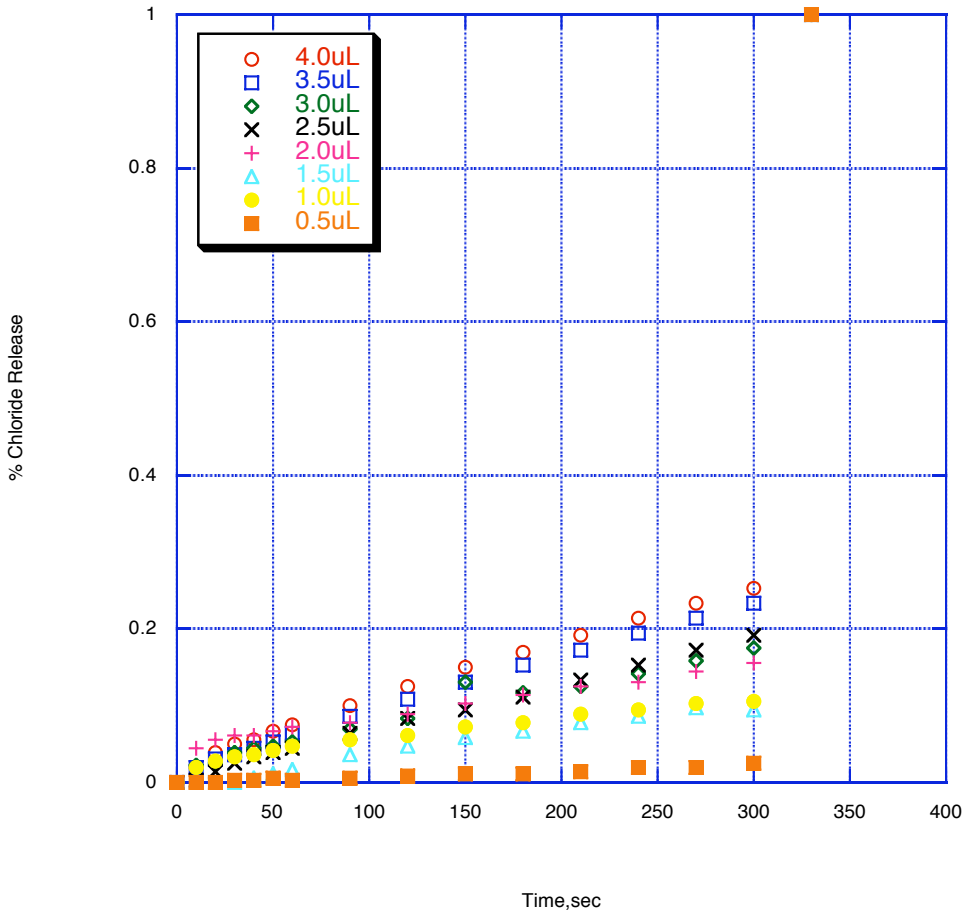


Figure 4.2 Chloride efflux induced by Carrier 4.5 at pH 7.4. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM by lipid concentration). The data point at 330 seconds (100% chloride release) was taken after the addition of the lysing agent, which released all of the entrapped chloride from the interior of the vesicles.

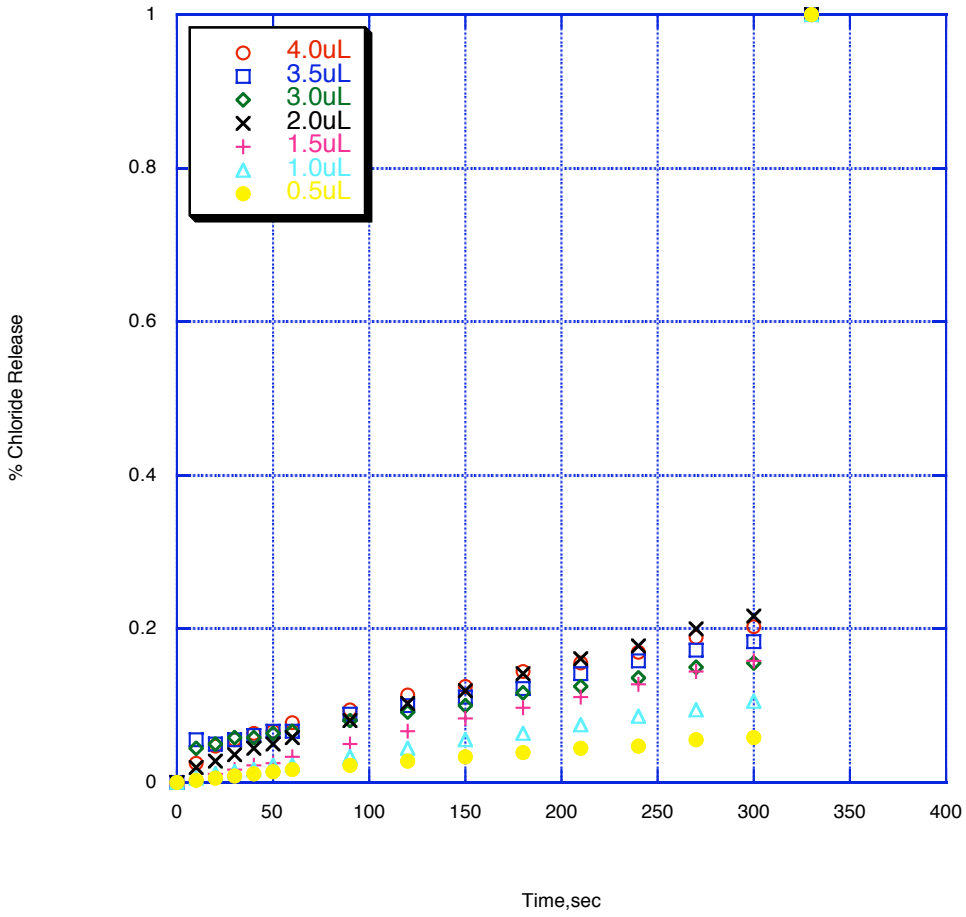
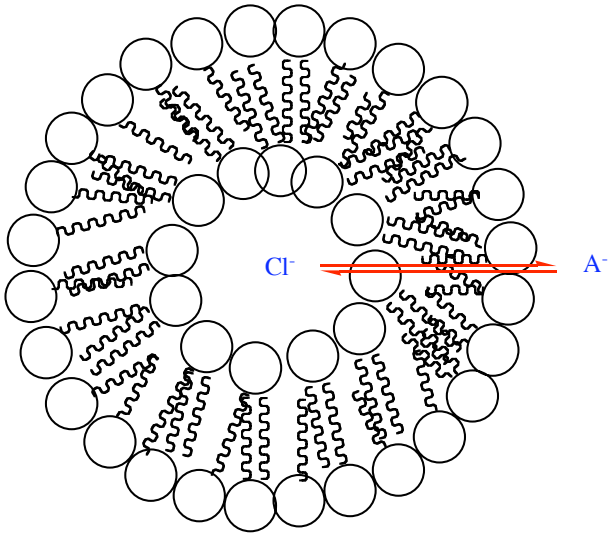
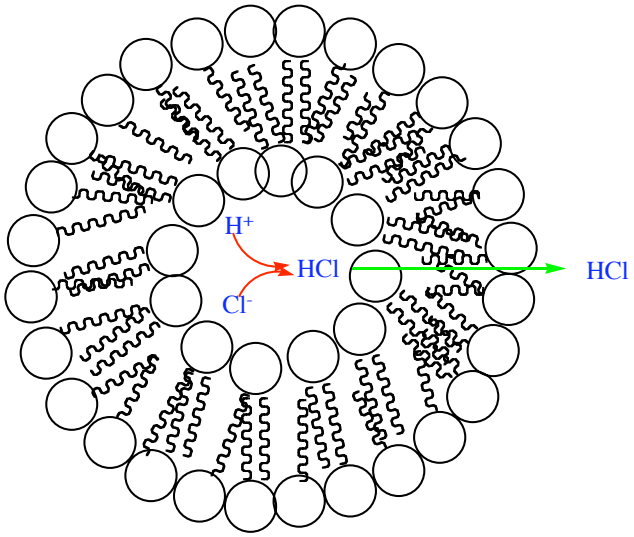


Figure 4.3 Chloride efflux induced by Carrier 4.5 at pH 5.5. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The data point at 330 seconds (100% chloride release) was taken after the addition of the lysing agent, which released all of the entrapped chloride from the interior of the vesicles.



antiport

Scheme 4.1 Antiport mechanism. The rate of chloride efflux from the interior is expected to be dependent on the identity of the external anion. The identities of the internal and external cations do not affect transport rates when transport occurs in accord with this mechanism.



Scheme 4.2 Symport mechanism. The rate of chloride efflux is expected to be independent of the identity of the external anion, and dependent on the identity of the internal cation. A net change in the internal pH of the liposomes is expected for transport that occurs via this mechanism.

4.4.3.2 Chloride transport rate with Carrier 4.6

Remarkably fast chloride release was demonstrated upon the addition of 0.5 μL of a 1 mM solution of prodigiosin in DMSO. Carrier **4.6** rapidly approached saturation in timescale of the experiment (Figure 4.4). The rate of chloride efflux due to Carrier **4.6** was taken as the benchmark against which the all of the experimental derivatives were compared. Chloride efflux was approximately 80%. The *in vivo* mechanism of action for natural prodigiosins is proposed to be H^+/Cl^- symport under biological conditions. The particular mode of action under these experimental conditions is discussed later in this chapter.

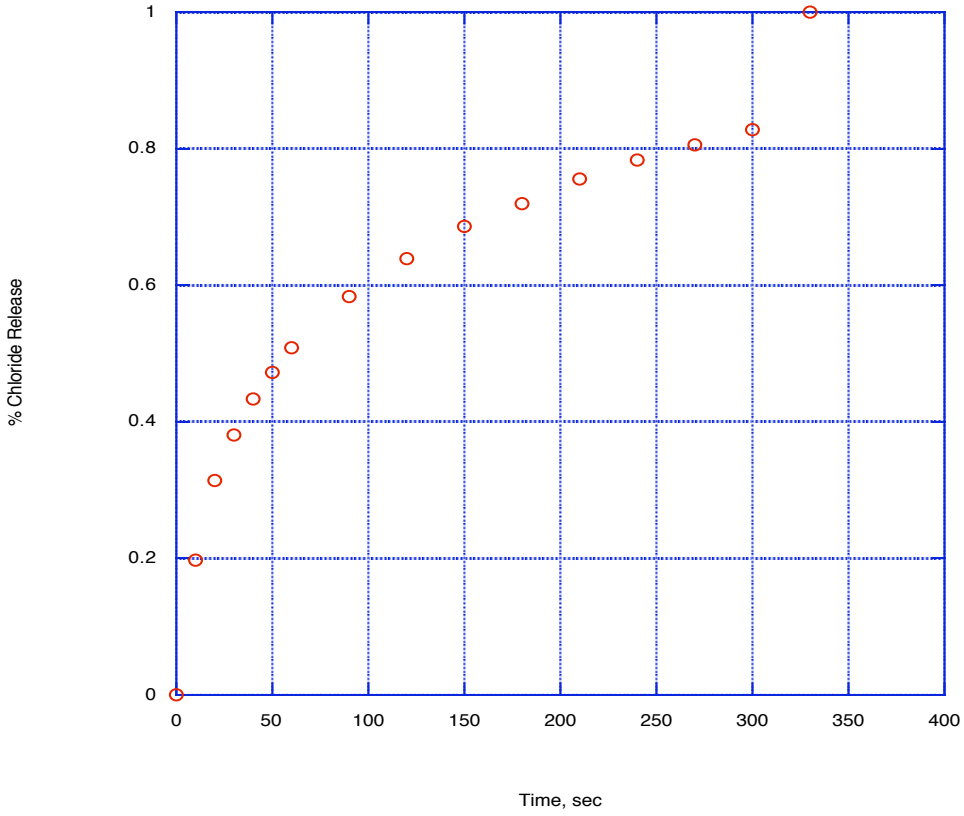


Figure 4.4 Chloride efflux induced by Carrier 4.6 (prodigiosin) at pH 7.4. The carrier was sufficiently active at the 0.5 μL addition level that higher concentrations were not examined. This efflux curve became the standard to which other measurements were compared. The endpoint (100% chloride release) was measured after addition of the lysing agent for this and all of the remaining efflux graphs.

4.4.3.3 Chloride transport rates with Carrier 4.7

The dipyrromethene (Carrier **4.7**) demonstrated some of the fastest rates of chloride efflux among the experimental derivates tested. The efflux rate was shown to be

linear at low concentrations (0.25 μL – 1.0 μL added) at pH 7.4. With increasing concentrations of the carrier, saturation was seen. Saturation became appreciable at added carrier amounts in excess of 1.5 μL (Figure 4.5). The maximal chloride efflux obtained was approximately 90%.

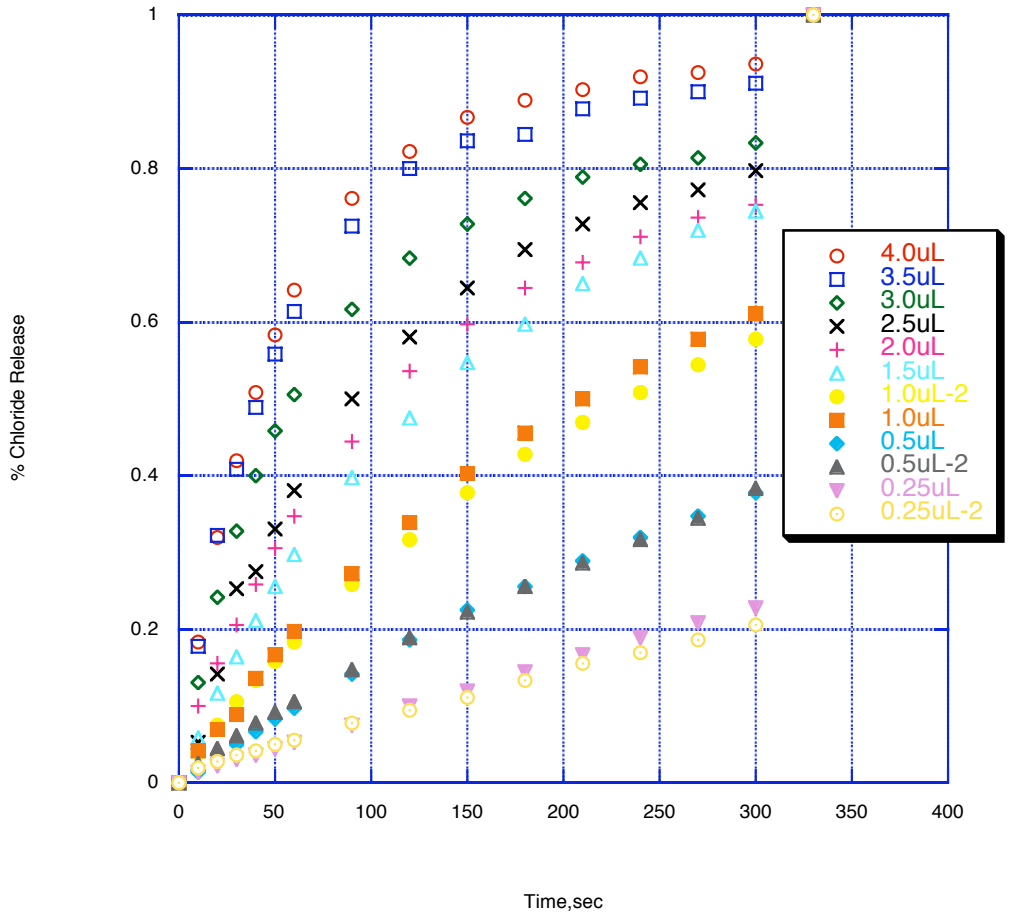


Figure 4.5 Chloride efflux induced by Carrier 4.7 at pH 7.4. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

At lower pH, no linear trends were seen. Further, strong evidence of saturation was observed at high carrier concentrations. Using 3.5 μL of **4.7**, no further change in the electrode reading was seen after 180 seconds until the liposomes were burst (Figure 4.6). The maximal chloride efflux obtained was approximately 90%.

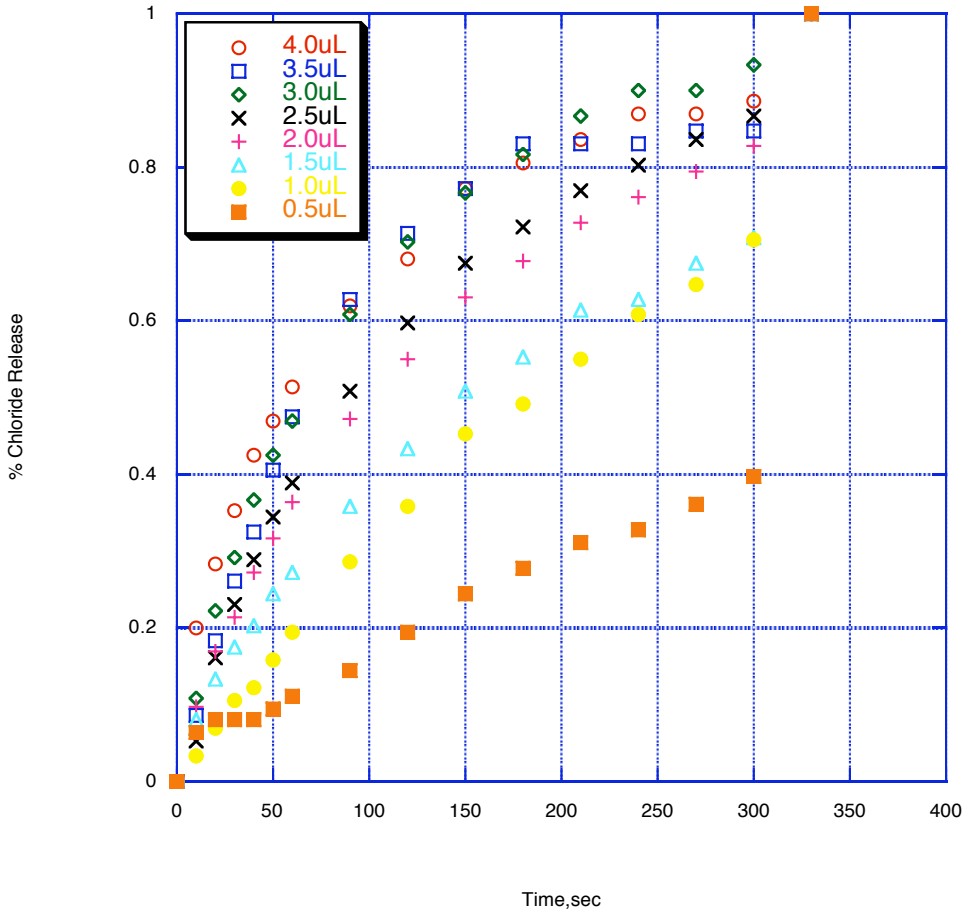


Figure 4.6 Chloride efflux induced by Carrier 4.7 at pH 5.5. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

4.4.3.4 Chloride transport rates with Carrier 4.8

The methoxy-dipyrromethene (Carrier **4.8**) displayed unusual behavior at pH 7.4. At low concentrations (1.0 μL – 2.0 μL added carrier) the rate of chloride efflux quickly saturated below 20% total release and did not change further. Addition of higher amounts of carrier resulted in more typical curves (Figure 4.7). These data trends were reproducible after several trials. The maximal chloride efflux obtained was approximately 60%.

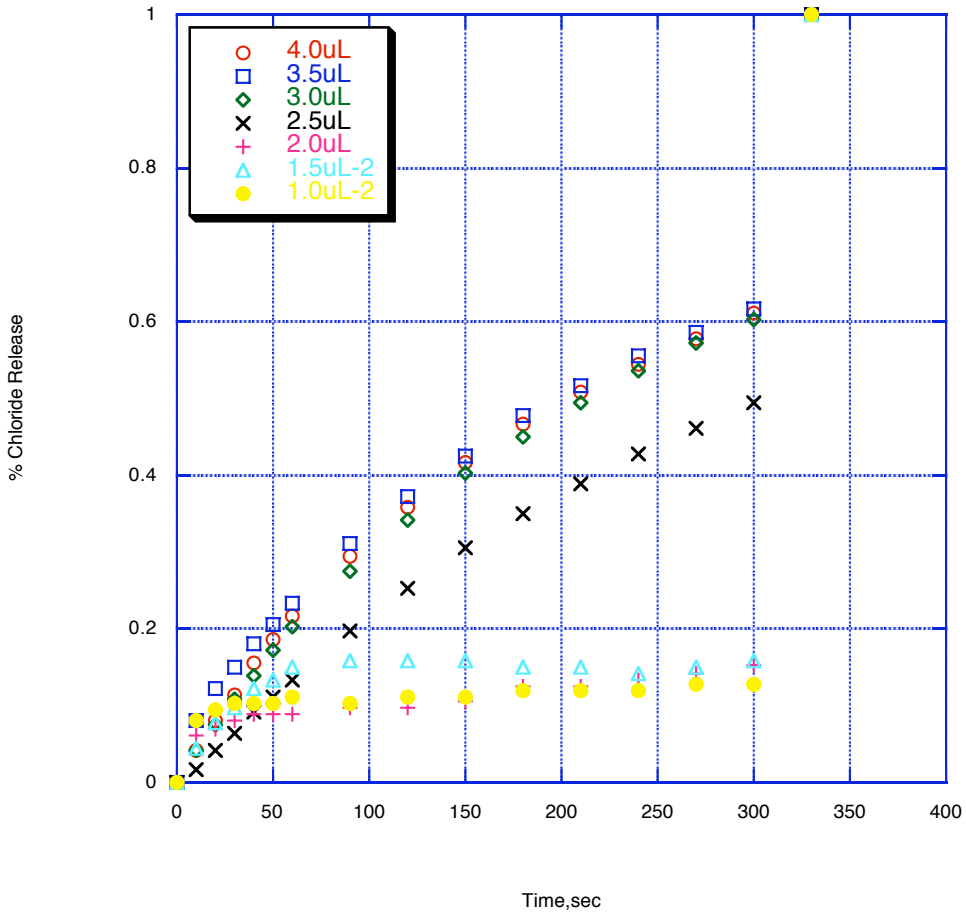


Figure 4.7 Chloride efflux induced by Carrier 4.8 at pH 7.4. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

Unlike the results at pH 7.4, the data for experiments conducted at pH 5.5 displayed no unusual characteristics. At low concentrations of carrier (0.5 μL and 1.0 μL), a linear trend was observed. At higher concentrations, the rates slowed after

approximately 150 seconds (Figure 4.8). The maximal chloride efflux obtained was approximately 85%.

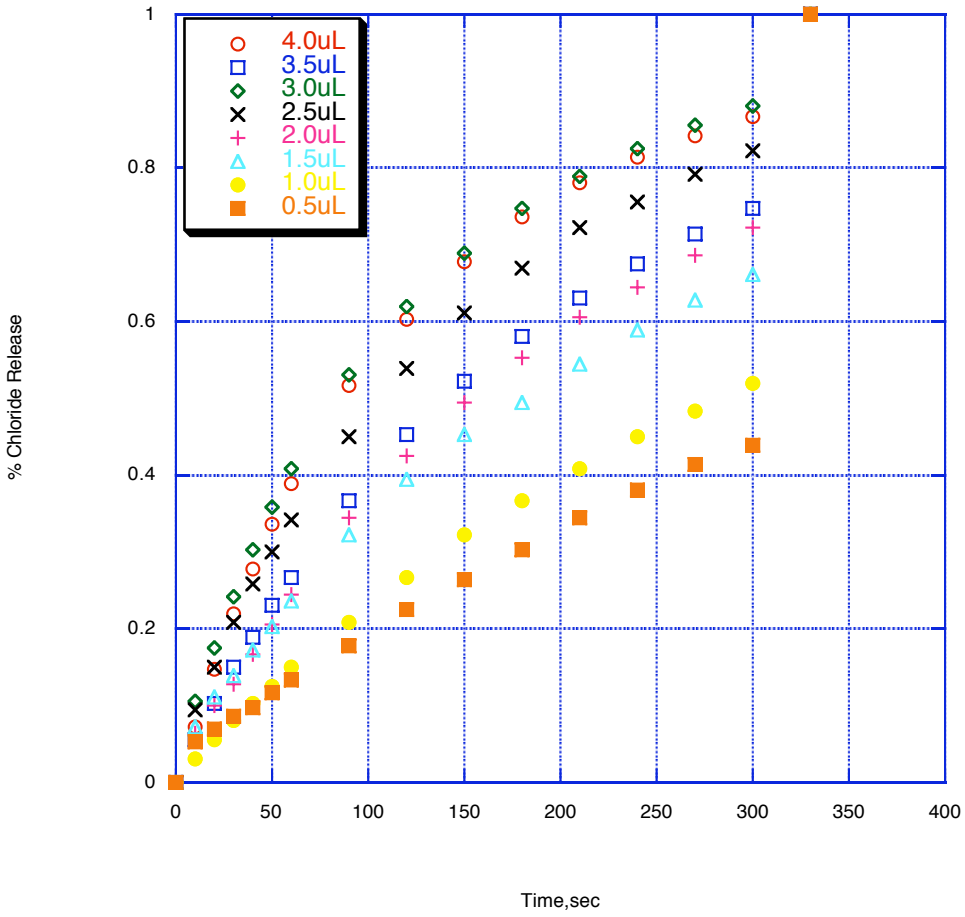


Figure 4.8 Chloride efflux induced by Carrier 4.8 at pH 5.5. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

4.4.3.5 Chloride transport rates with Carrier 4.9

Carrier **4.9**, a calixpyrrole bearing an appended urea group, is not a readily protonated species. Chloride efflux rates were modest for this molecule (between 10% and 15%) as shown in Figure 4.9. Unlike the easily-protonated species, should an H^+/Cl^- symport mechanism be at work, a pH gradient would certainly be established as the calixpyrrole could not readily give up a proton to the newly formed hydroxide in the liposomal interior. The details of mechanistic investigations are presented later in this chapter.

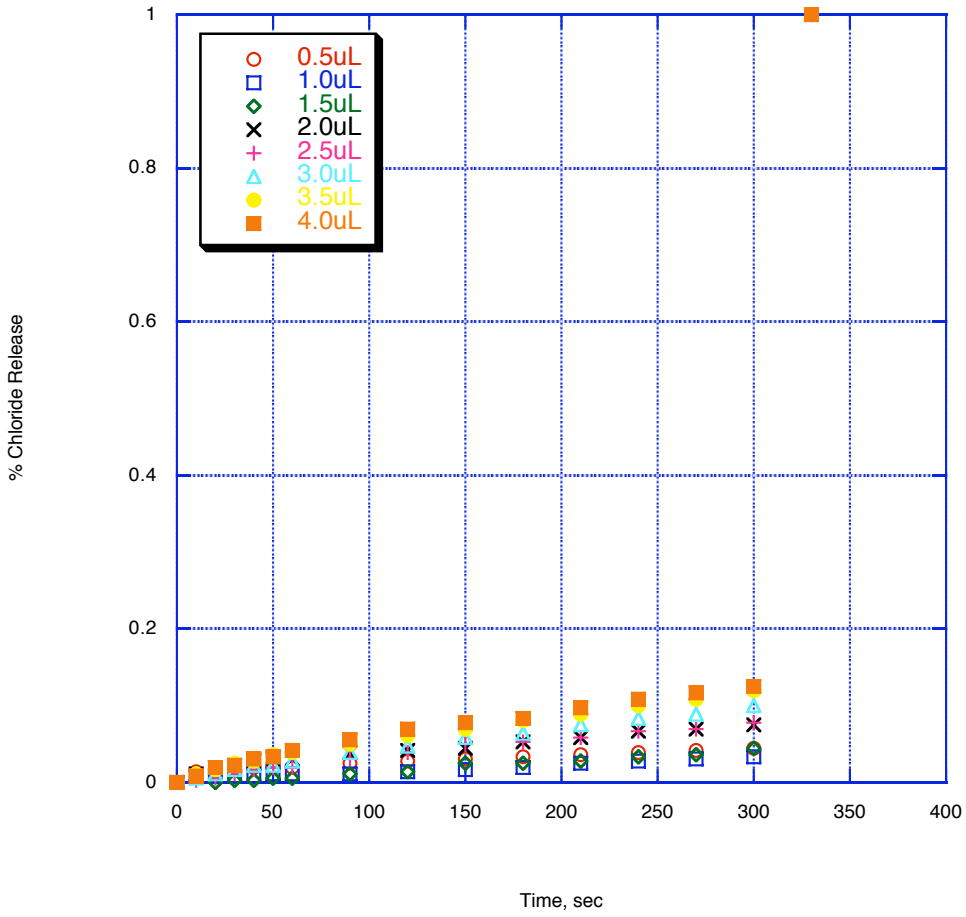


Figure 4.9 Chloride efflux induced by Carrier 4.9 at pH 7.4. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

The rates of chloride efflux that are seen at pH 7.4 are essentially unchanged at pH 5.5 (Figure 4.10).

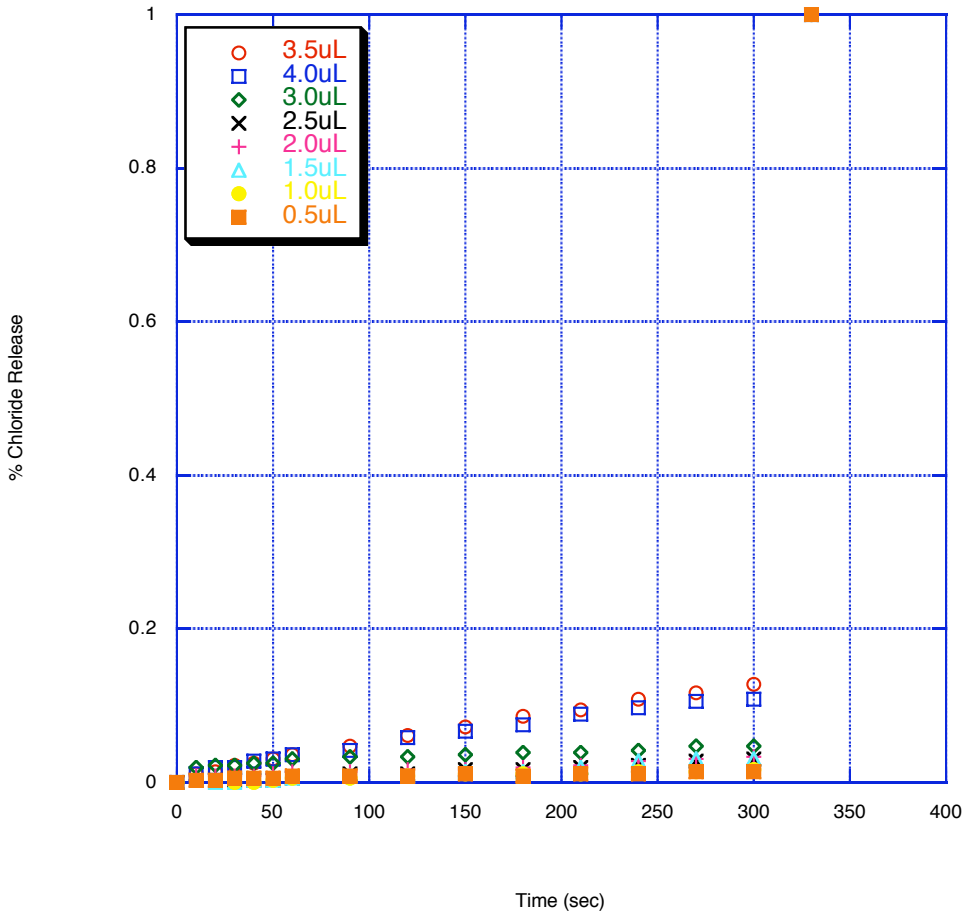


Figure 4.10 Chloride efflux induced by Carrier 4.9 at pH 5.5. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

4.4.3.6 Chloride transport rate with Carrier 4.10

This particular sapphyrin crashed out of solution upon addition to the aqueous liposome suspension. No transport data was available for this molecule. The more water-soluble sapphyrin, Carrier **4.11**, was substituted.

4.4.3.7 Chloride transport rates with Carrier 4.11

At pH 7.4, the water-soluble sapphyrin effected moderate release of chloride from liposomes; the percentage released did not exceed approximately 20% (Figure 4.11). Rates slowed within the first 30 seconds, with little additional change in the extent of chloride released after 100 seconds being observed at any carrier concentration used. The hydrophilicity of this particular sapphyrin species is believed to be responsible, in part, for the modest performance in the transport studies. This particular molecule was also the largest species examined. The sheer size of the molecule could be preventing it from navigating the denser regions of the lipid bilayer. Although there is no evidence at present that would serve to confirm or refute this latter hypothesis, it is clear that, on the whole, smaller species gave better transport results.

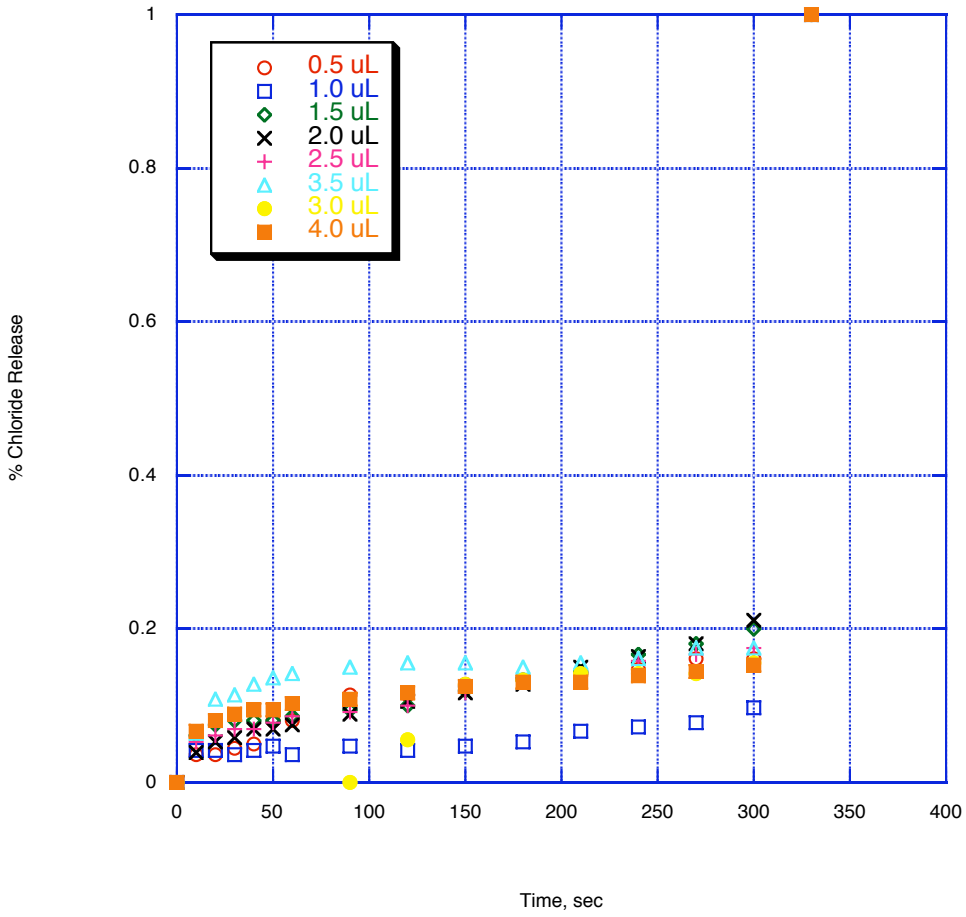


Figure 4.11 Chloride efflux induced by Carrier 4.11 at pH 7.4. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

At, pH 5.5, the overall chloride release was higher; approximately 40% (Figure 4.12). At lower concentrations, a linear relationship between the percentage of chloride released and time was observed. With increasing amounts of carrier, the relationship

became less linear. However, saturation behavior was not observed to any appreciable degree.

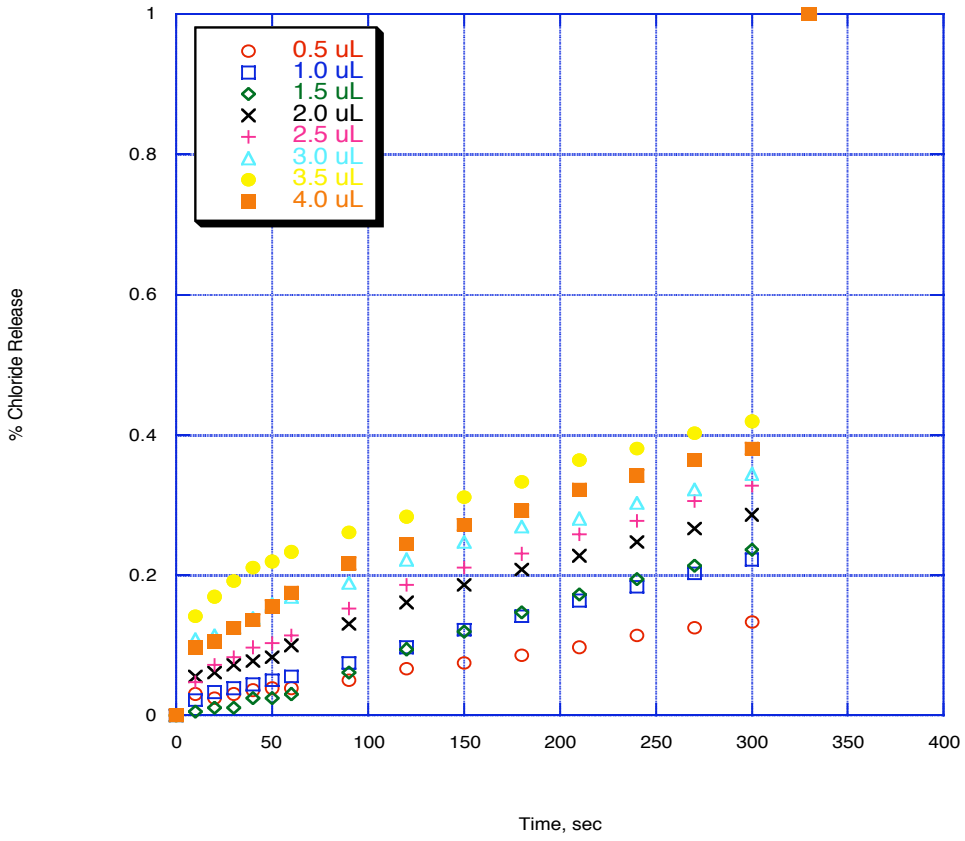


Figure 4.12 Chloride efflux induced by Carrier 4.11 at pH 5.5. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

4.4.3.8 Chloride transport rates with Carrier 4.12

The neutral terpyrrole did not effect chloride efflux to any significant degree at pH 7.4. Further studies with the molecule were discontinued in favor of more promising targets. A small amount of efflux was observed, i.e. less than 5% (Figure 4.13).

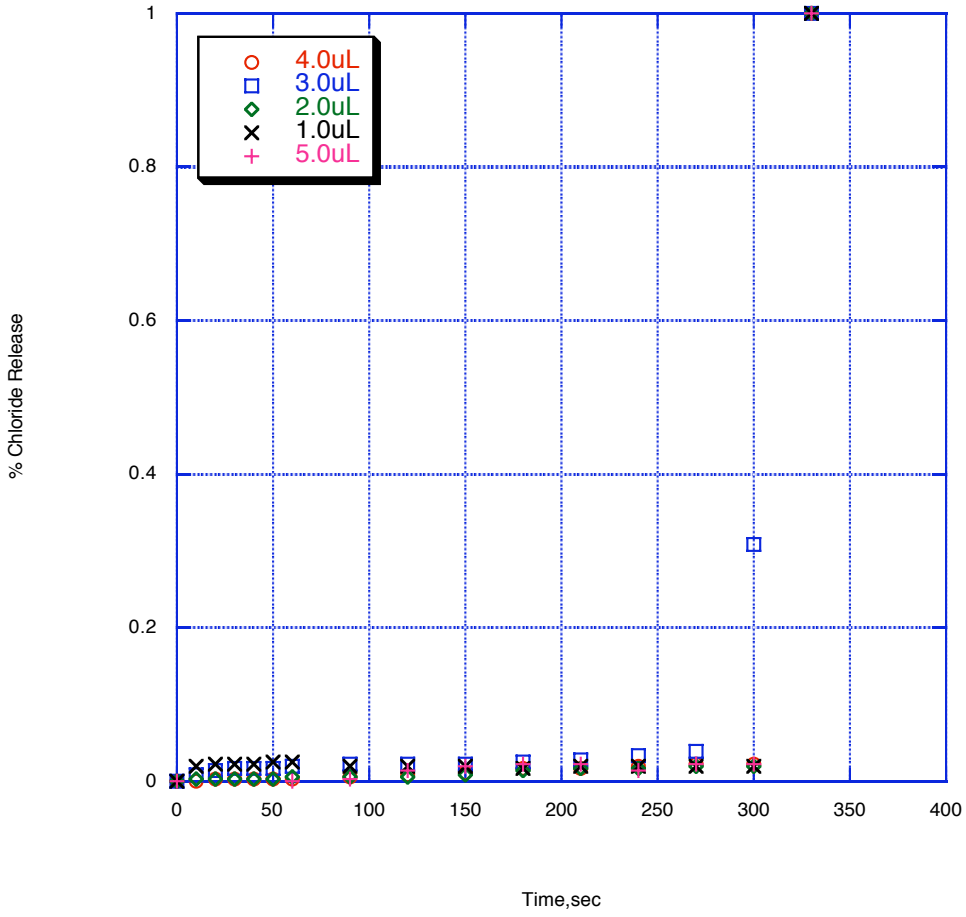


Figure 4.13 Chloride efflux induced by Carrier 4.12. Aliquots of a 1 mM DMSO solution of carrier were added in the amounts indicated to a 5.0 mL suspension of liposomes (1 mM in total lipid concentration). The endpoint at 330 seconds was measured after the addition of lysing agent.

4.4.3.9 Summary of carrier transport rates

A look at the maximal efflux rates for each carrier at pH 7.4 and pH 5.5 are shown in Figure 4.14 and Figure 4.15. In Figure 4.14, all of the maximal rates were recorded using 3.0 μL - 4.0 μL of added carrier, with the exception of Carrier **4.6**, the natural prodigiosin, in which case only 0.5 μL were used. Of the synthetic species tested, Carrier **4.7**, represented by the green open diamonds, a dipyrromethene derivative, induced the highest effluxes of chloride from the liposomal interiors. Carrier **4.8**, represented by the black x's, another dipyrromethene derivative also demonstrated notably higher efflux rates than the remainder of the molecules tested. The difference in the efflux rates for the two carriers was far less at pH 5.5 than at pH 7.4.

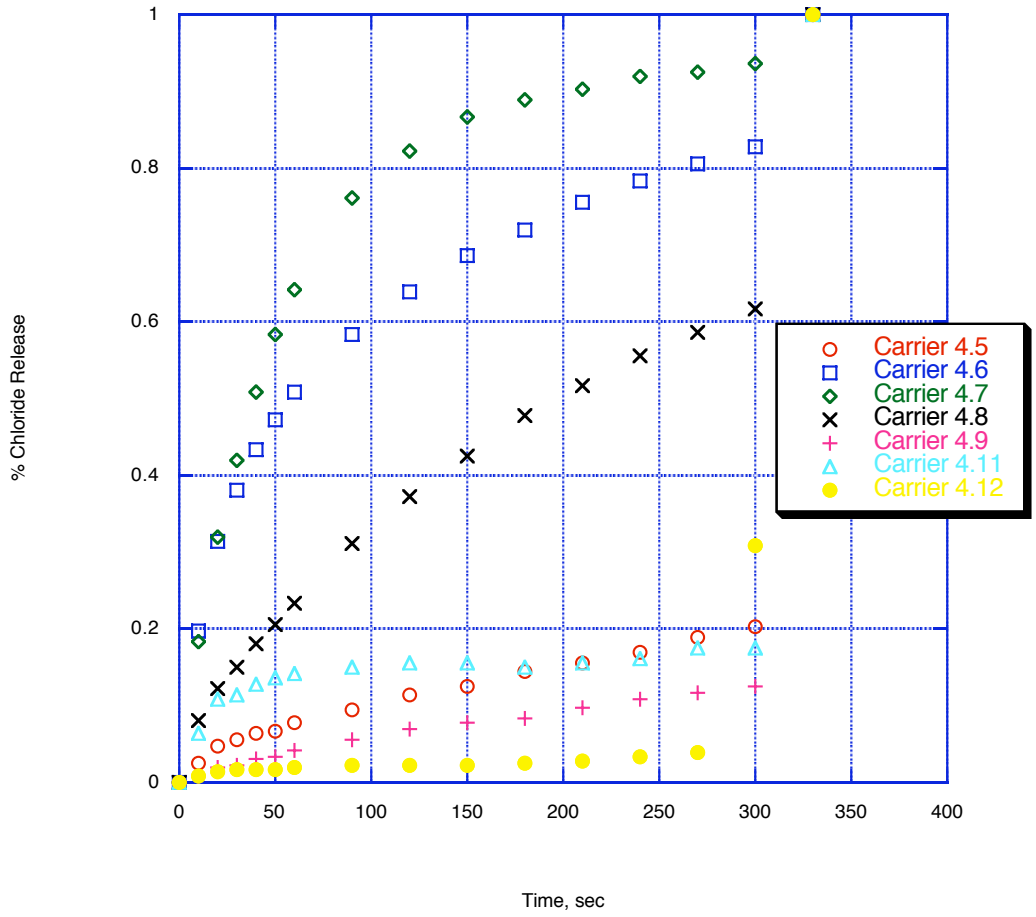


Figure 4.14 Maximal chloride efflux rates for each carrier at pH 7.4. Carrier **4.6**, the natural prodigiosin, was aliquoted at a 0.5 μL volume. The other carriers were aliquoted at 3.0-4.0 μL volumes to obtain maximal transport rates. The endpoint at 330 seconds was measured after the addition of lysing agent.

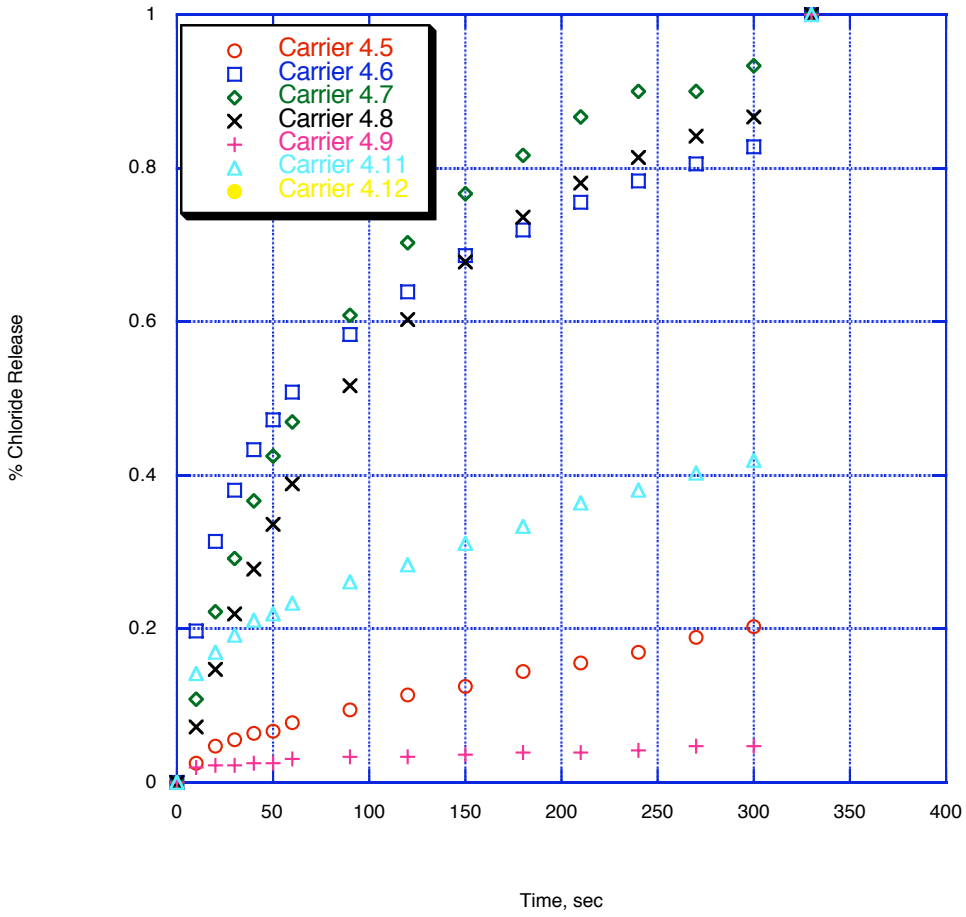


Figure 4.15 Maximal chloride efflux rates for each carrier at pH 5.5. Carrier **4.6**, the natural prodigiosin, is shown for comparison although the data curve in question was actually recorded at pH 7.4. Carrier **4.6** was aliquoted at a 0.5 μL volume, whereas the other carriers were aliquoted at 3.0-4.0 μL volumes.

4.4.3.10 Comparison between linear carriers

The dipyrromethenes were shown to be affected the most by changes to the pH of the suspension (Figure 4.16). For the molecules containing three pyrrole subunits, the

rates at each pH essentially overlapped with one another. As expected, the species that did not readily protonate, Carrier **4.12**, effected the least chloride efflux from the liposomes. Only in the case of Carrier **4.7** did the transport rates significantly dropped when the external pH was lowered. This could point to a symport mechanism at work. More detailed mechanistic studies are presented later in this chapter.

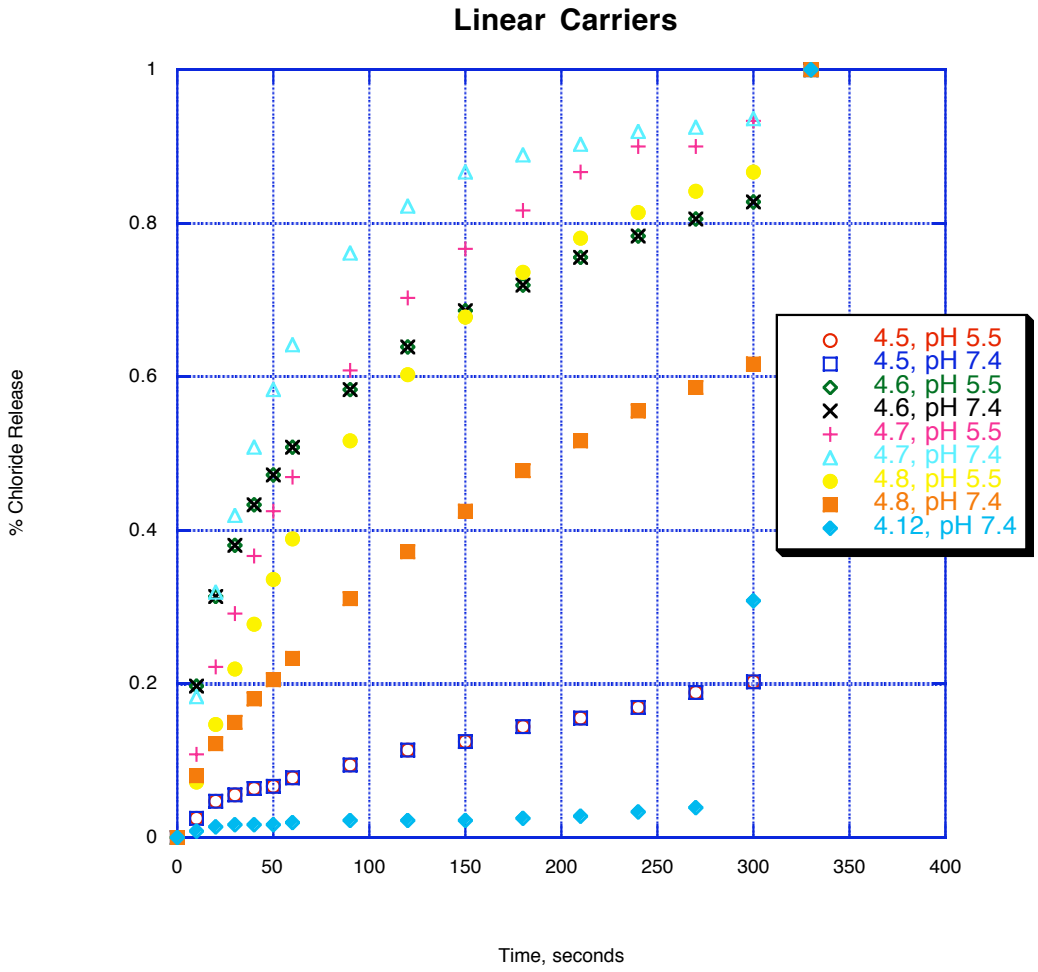


Figure 4.16 Maximal rates of chloride efflux induced by the linear carriers at pH 5.5 and pH 7.4. All carriers except **4.6** were aliquoted at 3.0-4.0 μL volumes. The endpoint at 330 seconds was measured after the addition of lysing agent.

4.4.3.11 Comparison between macrocyclic carriers

A somewhat surprising result was the relatively poor performance of sapphyrin, Carrier **4.11**, as an inducer of chloride efflux (Figure 4.17). At lower pH, Carrier **4.11** demonstrated fair efflux, approximately 40%. However, at pH 7.4, this sapphyrin performed similarly to calixpyrrole, Carrier **4.9**, with the caveat that **4.11** saturated and **4.9** exhibited linear behavior. Experiments conducted for longer periods of time might show calixpyrrole overtaking sapphyrin and demonstrating more enhanced chloride efflux efficiencies. The fact that the transport rates for sapphyrin increased with at lower pH could be indicative of an antiport mechanism at work. At a lower external pH, a symport mechanism would be hindered, given that further raising the pH gradient across the membrane would be energetically unfavorable. By the same reasoning, the fact that the transport rates for calixpyrrole dropped when the external pH was lowered, could be indicative of a symport mechanism at work. Additional mechanistic studies for these molecules are presented later in this chapter.

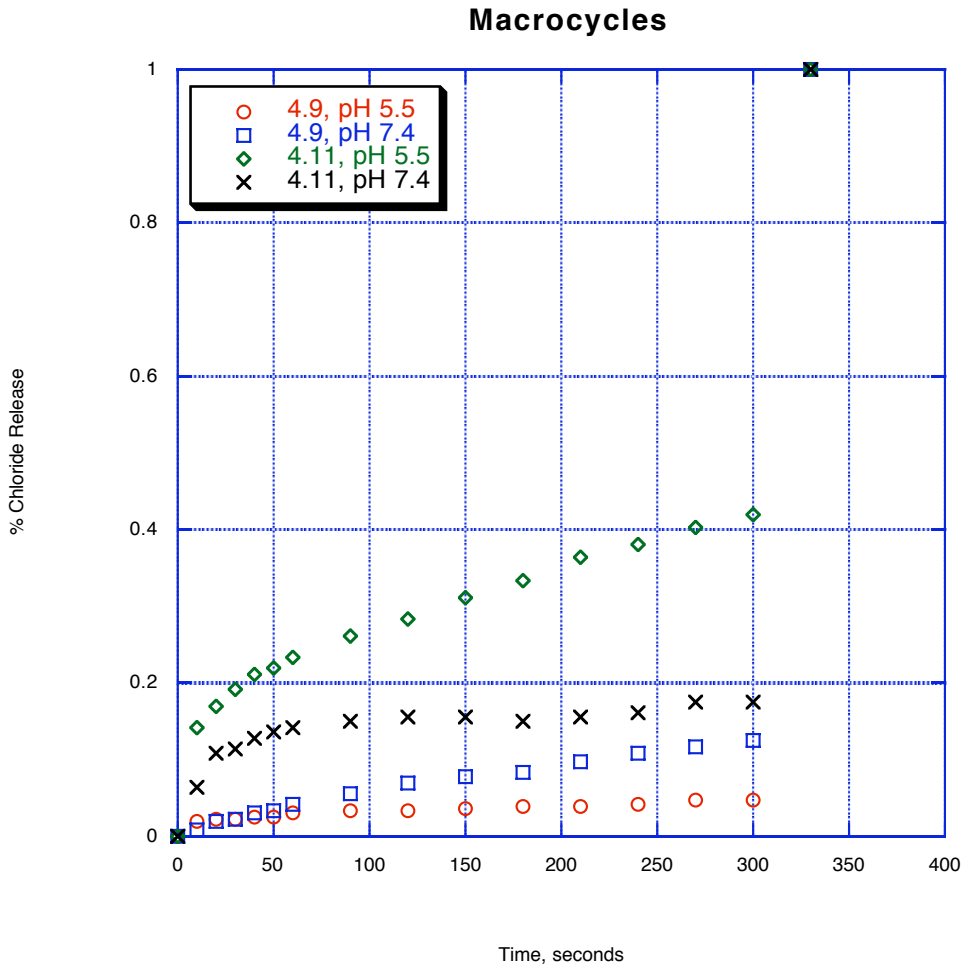


Figure 4.17 Maximal rates of chloride efflux induced by macrocyclic carriers at pH 5.5 and pH 7.4. The carriers were aliquoted at 3.0-4.0 μL volumes. The endpoint at 330 seconds was measured after the addition of lysing agent.

4.5 Results of Mechanistic Studies

In order to determine by which mechanism each carrier molecule was acting, additional chloride efflux studies were carried out in which the conditions were altered to favor an antiport mechanism. The external anion was changed from nitrate to sulfate, a larger and harder ion, which would be less likely to cross the membrane as part of an

antiport mechanism. Molecules that transported chloride via a symport mechanism would be expected to be unaffected by this change. However, molecules that transported chloride via a symport mechanism would be expected to exhibit a decrease in chloride transport under these new conditions.

A second study was performed in which liposomes were prepared with a pH-sensitive dye entrapped with NaCl inside. Molecules that transported chloride via an H^+/Cl^- symport mechanism would cause a net buildup of hydroxide ion inside the liposomal interior and alter the fluorescence of the entrapped dye. Molecules that transported chloride via an antiport mechanism would not effect any changes in the fluorescence spectrum of the entrapped dye. It should be noted that for these two mechanism studies, no other changes in experimental conditions were made.

As a third test of the mechanistic behavior of the molecules, again dye-loaded vesicles were prepared. In this study, nitrate was used as the external counter ion, which is favorable for an antiport mode of action. Additional hydroxide was added to the liposomal suspension to create a pH gradient across the membrane and facilitate an H^+/Cl^- mechanism. Under these competing conditions, the behavior of each carrier was observed to determine if the mechanism of transport could be switched from antiport to symport.

4.5.1 Antiport mechanism studies

These experiments were carried out in exactly the same manner as the chloride transport rate studies that were previously discussed, with the exception that 500 mM Na_2SO_4 was used instead of $NaNO_3$ as the external salt. 2.0 μL aliquots of carrier solutions (5 mM in DMSO) were used in these experiments. Figure 4.18 summarizes the transport rates that resulted from switching the counter ion to sulfate. Prodigiosin,

Carrier **4.6**, not surprisingly, shows the highest chloride transport ability under these conditions. Case by case comparisons for each carrier under both sets of conditions are shown in Figures 4.19-4.25.

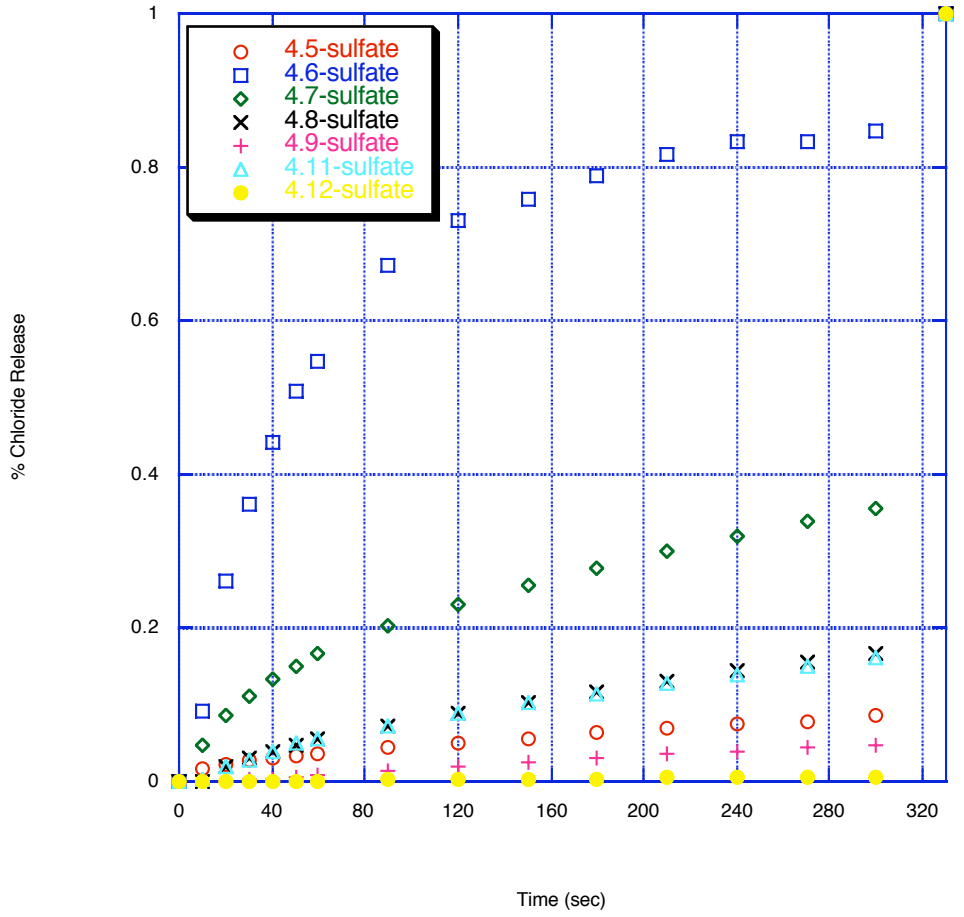


Figure 4.18 Summary of antiport mechanism experiments. 2.0 μL aliquots of 5 mM carrier solutions in DMSO were added to 5.0 mL liposome suspensions in 500 mM aqueous sodium sulfate solution.

4.5.1.1 Carrier 4.5

The chloride transport ability of Carrier **4.5** dropped off noticeably by changing the external anion from the more transportable nitrate to the larger, harder sulfate anion (see Figure 4.19). These data support an antiport mechanism in which the carrier is initially binding nitrate, which is then transported into the interior of the liposome, where it is exchanged for chloride anion. The fact that transport rates increased as the pH was lowered, as was discussed previously, further supports the possibility of an antiport mechanism.

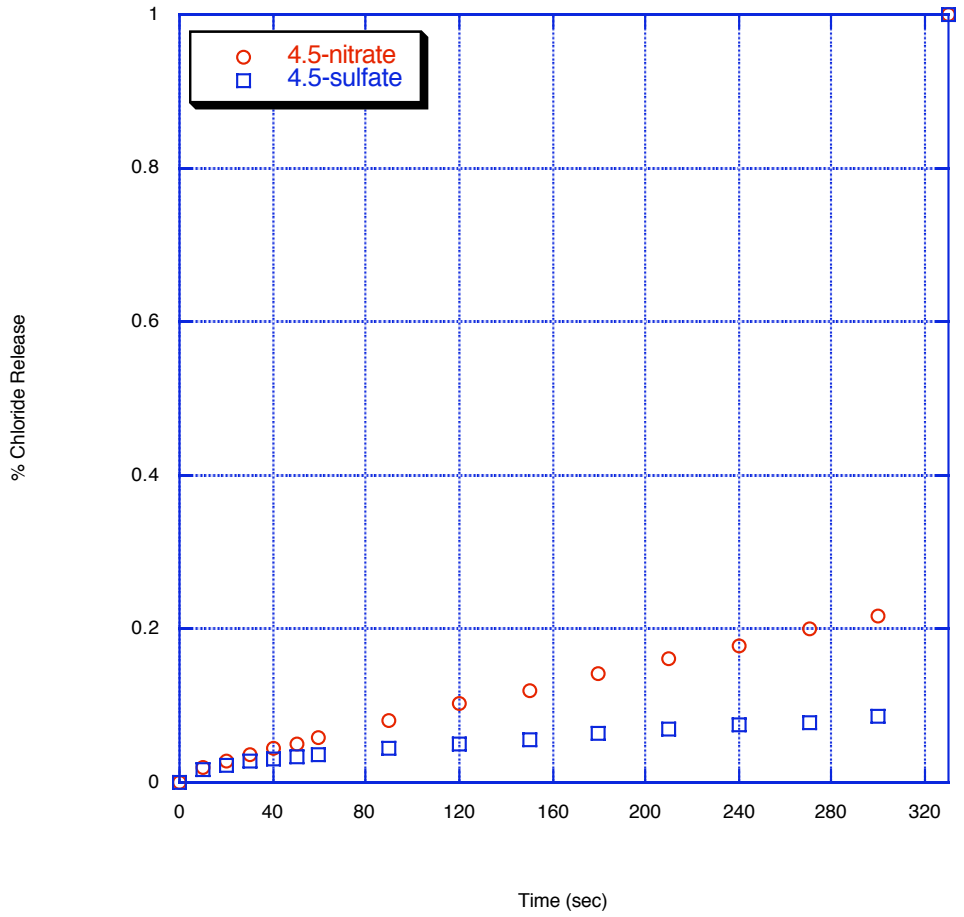


Figure 4.19 Carrier 4.5 antiport mechanism study. 2.0 μL of a 5 mM solution were added to liposome suspensions in 500 mM $NaNO_3$ and Na_2SO_4 solutions.

4.5.1.2 Carrier 4.6

Less overall change was seen when the external anion was changed from nitrate to sulfate in the case of prodigiosin **4.6**. Within the first 120 seconds, the rates were lower, however, higher levels of transport were achieved under the sulfate conditions over time (see Figure 4.20). This result does not conclusively answer the mechanism

question. In the previously discussed pH studies, identical transport rates were achieved for pH 5.5 and pH 7.4, which could support a symport mechanism. Again, these data are not conclusive.

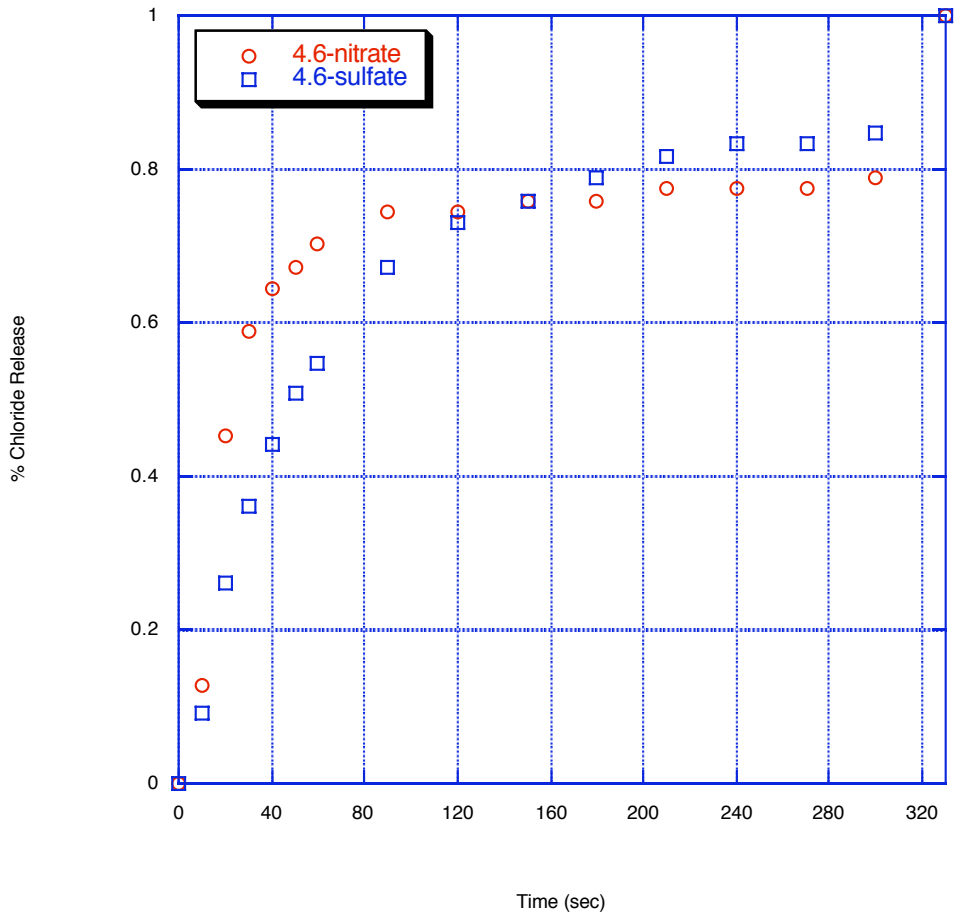


Figure 4.20 Carrier 4.6 antiport mechanism study. 2.0 μL of a 5 mM solution were added to liposome suspensions in 500 mM $NaNO_3$ and Na_2SO_4 solutions.

4.5.1.3 Carrier 4.7

Chloride transport rates significantly drop off when sulfate is substituted for nitrate as the external anion.

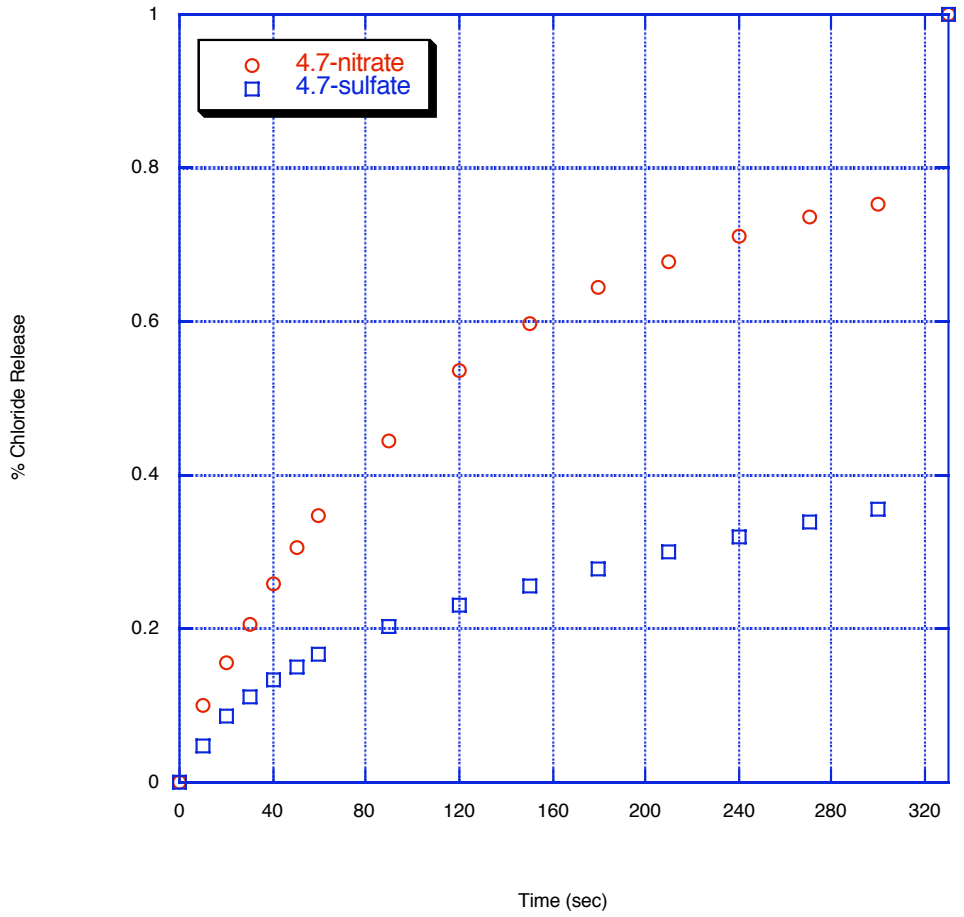


Figure 4.21 Carrier 4.7 antiport mechanism study. 2.0 μL of a 5 mM solution were added to liposome suspensions in 500 mM $NaNO_3$ and Na_2SO_4 solutions.

4.5.1.4 Carrier 4.8

Despite the results of the pH-dependent experiments, which would argue for an antiport mechanism, the lack of significant change in the transport rates when the external anion was changed, seemingly does not argue for the same mechanism. Considering the unusual behavior of Carrier **4.8** that was discussed previously, namely that at additions below 3.0 μL, the transport efficiency remained low (< 20%), and at additions above 3.0 μL, the transport rates increased significantly, it is not clear that the lack of significant changes shown in Figure 4.22 (in which the experiments were conducted using 2.0 μL aliquots of carrier solution) argue for a symport mechanism.

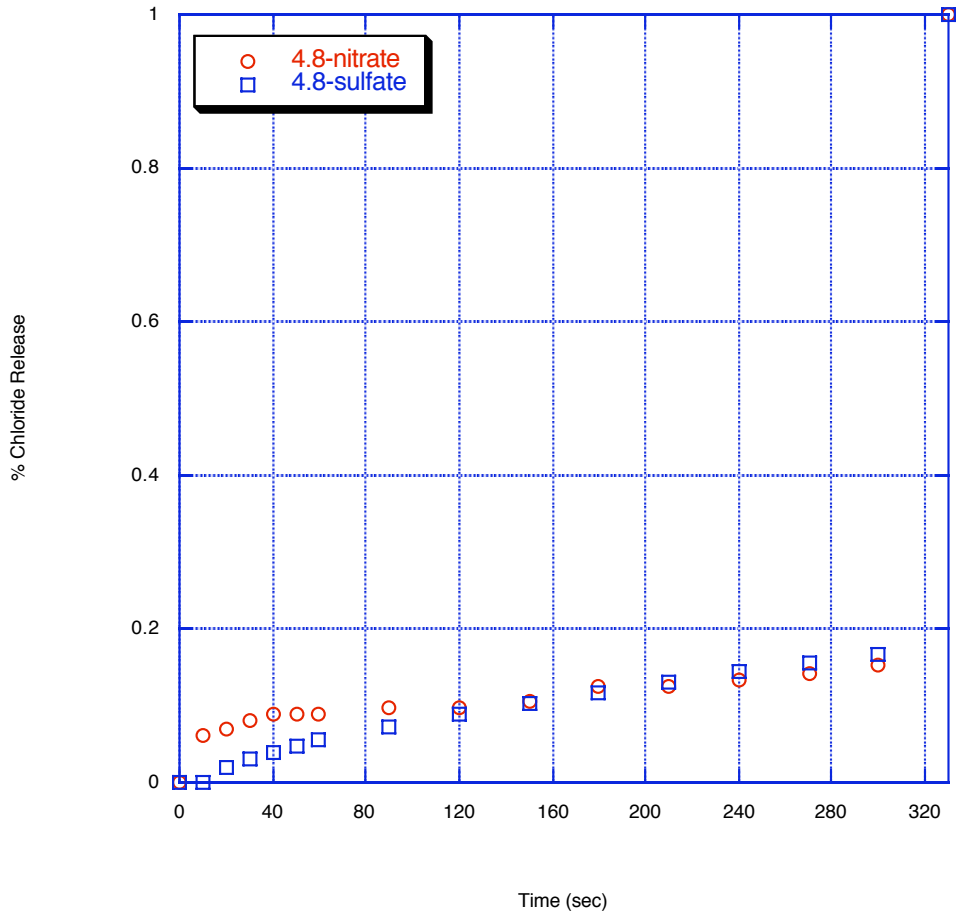


Figure 4.22 Carrier 4.8 antiport mechanism study. 2.0 μL of a 5 mM solution were added to liposome suspensions in 500 mM $NaNO_3$ and Na_2SO_4 solutions.

4.5.1.5 Carrier 4.9

The transport rates for the neutral calixpyrrole were shown to not be significantly affected by substituting the counter anion. This result, coupled with the result of the pH studies (transport dropped as the external pH dropped), does not support an antiport mechanism.

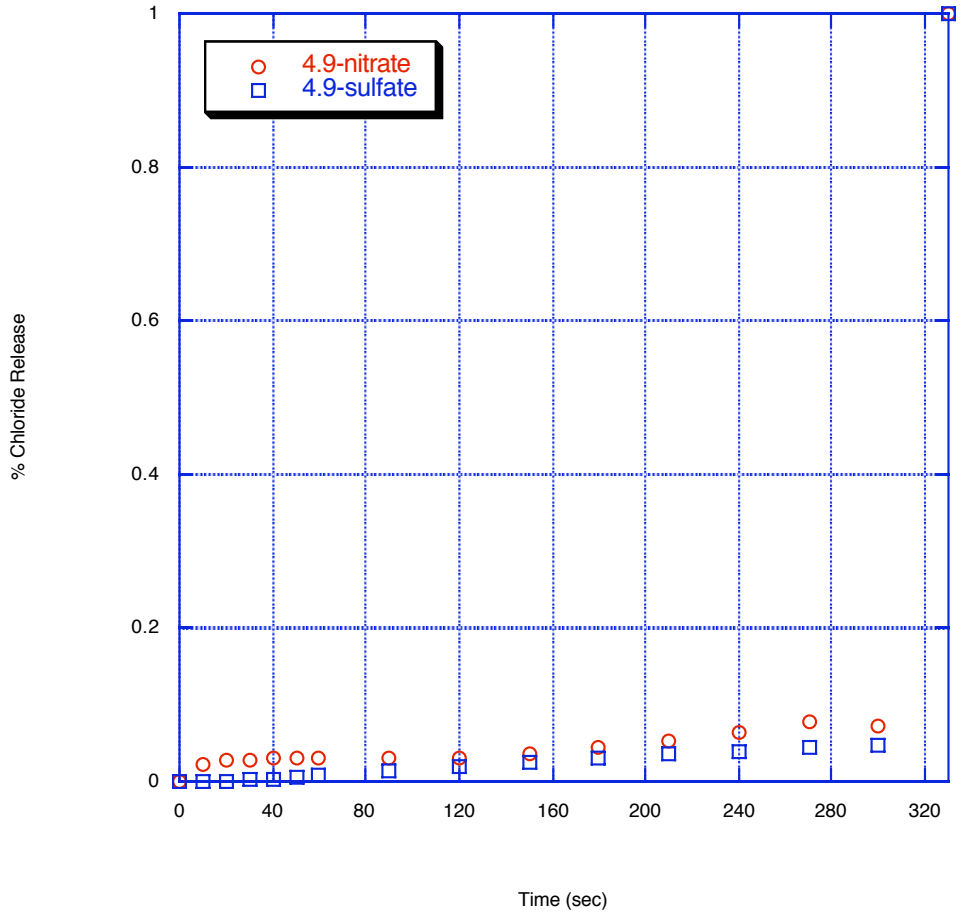


Figure 4.23 Carrier 4.9 antiport mechanism study. 2.0 μL of a 5 mM solution were added to liposome suspensions in 500 mM $NaNO_3$ and Na_2SO_4 solutions.

4.5.1.5 Carrier 4.11

Sapphyrin **4.11** appeared to be relatively unaffected by substituting sulfate for nitrate as the external anion. The lack of significant change would not indicate a strong antiport mechanism, however, it does not positively conclude a symport mechanism either. The chloride transport rates increased when the external pH was lowered from 7.4

to 5.5. That particular result might argue for an antiport mechanism. Experiments using a pH-sensitive dye were performed to further shed light on the mode of transport for this molecule.

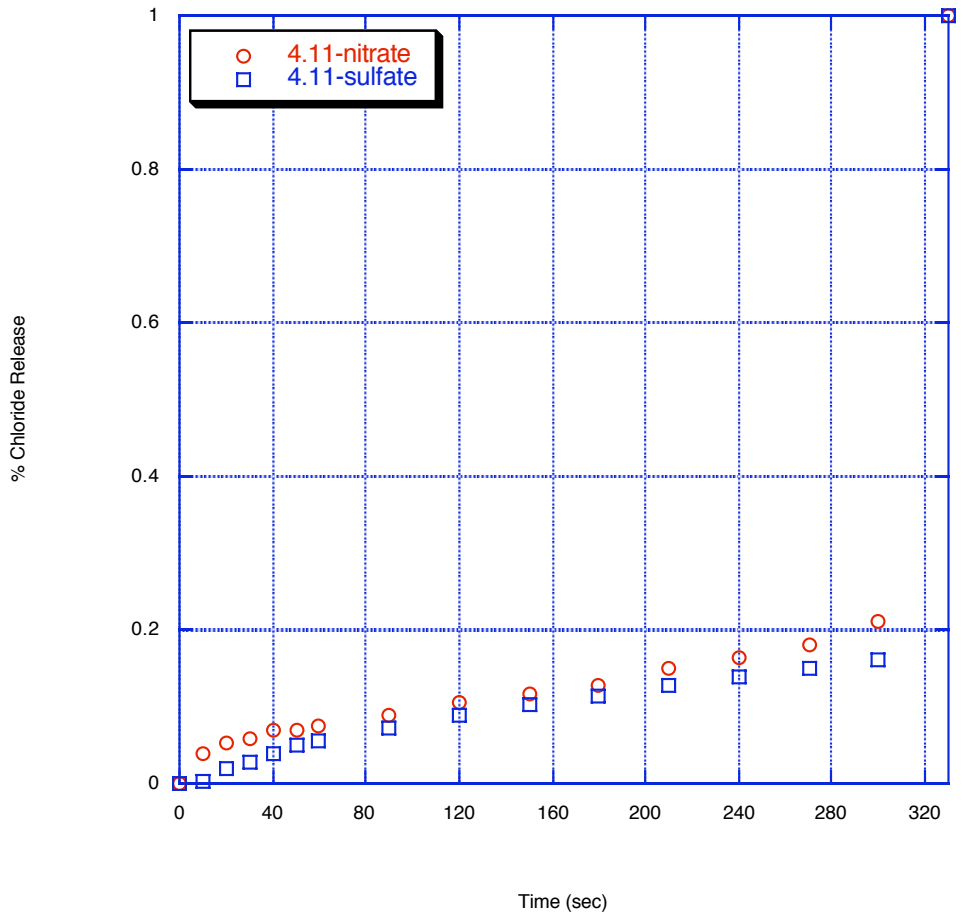


Figure 4.24 Carrier 4.11 antiport mechanism study. 2.0 μL of a 5 mM solution were added to liposome suspensions in 500 mM $NaNO_3$ and Na_2SO_4 solutions.

4.5.1.6 Carrier 4.12

In the case of the terpyrrole **4.12**, the chloride transport was extremely low initially (see Figure 4.25). The fact that the transport rate did not drop further when the counter anion was changed to sulfate, may not indicate a symport mechanism. This result may only reflect the low overall transport efficiency of Carrier **4.12**.

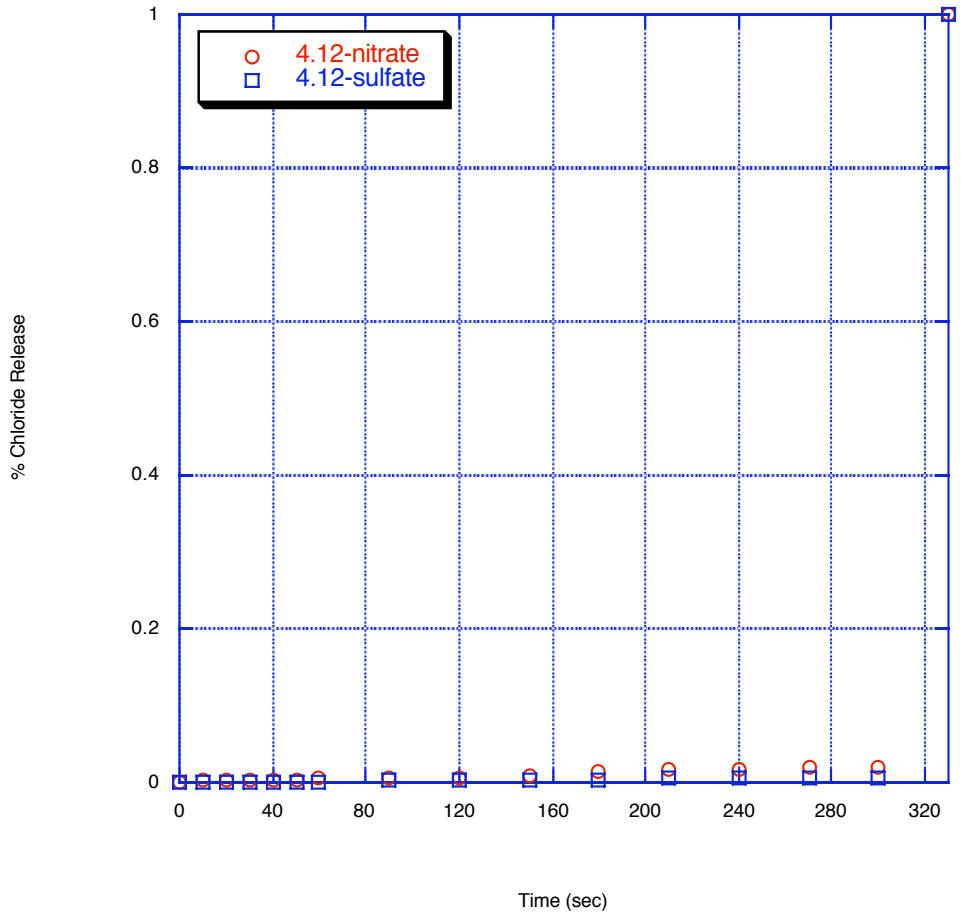


Figure 4.25 Carrier 4.12 antiport mechanism study. 2.0 μL of a 5 mM solution were added to liposome suspensions in 500 mM $NaNO_3$ and Na_2SO_4 solutions.

4.5.2 Symport mechanism studies using HPTS

Although a negative antiport mechanism result could suggest that a given carrier transports chloride via a symport mechanism, additional experiments were carried out to better determine whether or not H^+/Cl^- was indeed the mode of actions for some of the carrier species. To this end, liposome suspensions were prepared in which 8-hydroxy-1,3,6-pyrene-trisulfonate (HPTS) were entrapped in the vesicular interior in addition to NaCl. HPTS is a pH-sensitive fluorescent dye. Over a wide range of pH values, this dye has an emission maximum at 511 nm. As the pH is lowered, the emission changes in intensity and wavelength. The excitation maxima for HPTS are 450 (at pH values 7-8) and 405 (pH below 6). Excitation ratio experiments were performed in which the sample was excited at both 405 nm and 450 nm, while monitored for emission intensity at 511 nm. In the case of molecules that transport chloride via a NO_3^-/Cl^- antiport mechanism, no changes in the spectra would be expected. In the case of molecules that transport chloride via an H^+/Cl^- symport mechanism, a change in the excitation ratio is expected as the pH inside the liposomal interior changes. Upon lysis of the liposomes, the fluorescence of HPTS is expected to be restored to its original spectrum when there is no external NaOH present and is expected to drop further when there is external NaOH present.

To a greater or lesser degree, all of the molecules under investigation behaved in the same manner. Following addition of the carrier solutions, a jump in the 450 nm spectral line and a drop in the 405 nm spectral line were seen. Upon lysis of the vesicles, the respective increase (450 nm) and decrease (405 nm) became even more pronounced. Additionally, dereasing the concentration of entrapped chloride resulted in a decrease in the pH change inside the vesicles. These experiments are the most clear suggestion of the

mode of transport; that suggestion being an H^+/Cl^- symport mechanism. A typical data set are shown below.

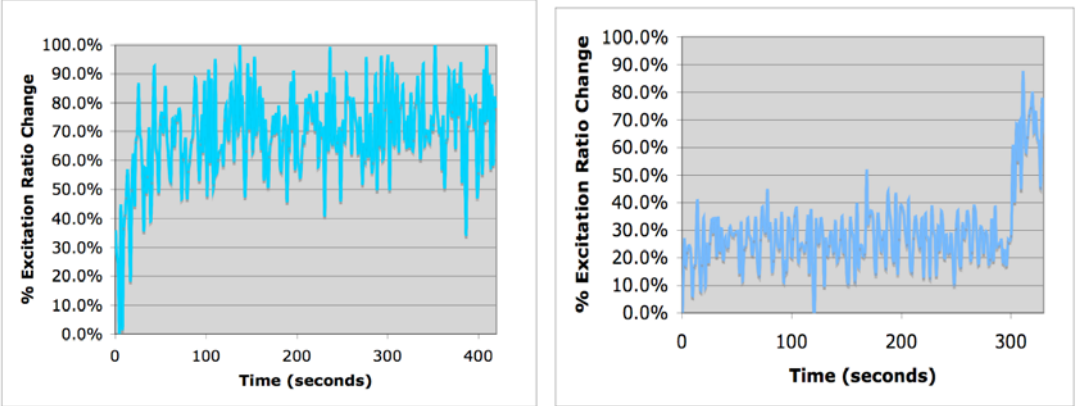


Figure 4.26. $H^=/Cl^-$ symport mechanism study using HPTS. (Left) Excitation ratio changes using Carrier **4.7** and vesicles containing 500 mM NaCl. (Right) Excitation ratio changes using Carrier **4.7** and vesicles containing 0.5 mM NaCl.

4.6 CONTROL STUDIES

4.6.1 Leakage results

To assure the experimental carriers were indeed inducing a transport event, rather than causing degradation of the membrane, control experiments were carried out using fluorescent dye-loaded liposomes. Liposomes were prepared with 0.5 mM carboxyfluorescein entrapped inside the vesicles in a manner analogous to the preparations that have been previously described. The liposome suspension was purified by gel-permeation chromatography to remove unincorporated dye. The liposomes eluted at a much faster rate than the unentrapped dye and were collected from the column, diluted, and used immediately. Suspensions of liposomes were monitored for a change in their fluorescence intensity as a result of addition of the carrier species. An increase in

fluorescence intensity was taken as leakage due to membrane tears induced by the experimental carriers.

None of the carriers examined were found to induce significant amounts of carboxyfluorescein leakage from the interior of the dye-loaded liposomes. On the basis of this control experiment, it was concluded that the observed chloride effluxes were due to transport events, rather than to random leakage.

4.6.2 Carrier partitioning results

Partitioning experiments were performed in a manner analogous to those discussed in Chapter 3. Carriers were dissolved in chloroform and contacted with an aqueous buffer solution for one hour under rotation. The phases were then separated and examined by UV-visible spectroscopy for color content. As opposed to the earlier studies, the distribution ratio was reversed. The distribution ratios were determined by diving the corrected aqueous absorbance by the corrected organic absorbance to determine the extent of the contamination of the aqueous phase by the carrier molecules.

At the concentrations examined for the transport studies, none of the carriers examined in these control studies partitioned to a high degree into aqueous solution. The water-soluble sapphyrin, Carrier **4.11** could not be subjected to this control study due to lack of sufficient material. However, given the design of the species and the modest performance in the transport experiments, it is believed that the molecule did partition significantly into the aqueous phase. This presumed preference is believed to be responsible for, at least in part, for the modest transport rates observed.

4.7 CORRELATION OF CHLORIDE EXTRACTION AND TRANSPORT

Summarizing the chloride extraction data given in Figure 3.10 and Table 3.1, the order of extraction efficiency was organic-soluble sapphyrin > prodigiosin derivative > dipyrromethene >> prodigiosin > hybrid calix-macrocycle. That order does not correspond completely with the chloride transport data discussed in this chapter. The order of transport efficiency for chloride presented in this chapter was prodigiosin >> dipyrromethene > methoxy-dipyrromethene > water-soluble sapphyrin > prodigiosin derivative > urea-appended calixpyrrole > Terpyrrole. Among the subset of species studied by both techniques, the correlation of binding, extraction and transport data are summarized in Table 4.1. Anion binding strength and anion transport efficiency do not seem to have a direct correlation at first glance. Carrier **4.6** has both a high binding constant (586,300 M^{-1}) and a high extraction ability (82%), however Carrier **4.9**, displays very modest transport (5%) despite having a high binding affinity for chloride (480,000 M^{-1}). However, there are other factors to be considered when comparing these molecules. Carrier **4.9** is a neutral species that would need to transport HCl using only hydrogen bonding interactions, whereas Carrier **4.6** can protonate and form an ionic bond to Cl^- for transport. Clearly, it can be seen from the data that ionic bonding results in greater chloride transport ability. Among the carriers possessing imine-like nitrogens, which could therefore protonate readily, there does seem to be some correlation between binding affinity and transport ability. It is less clear that there is a direct relationship between extraction and transport ability.

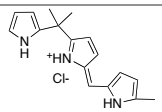
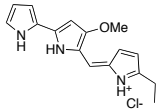
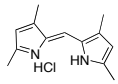
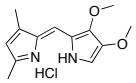
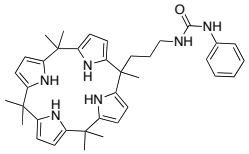
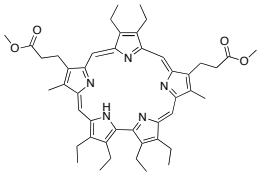
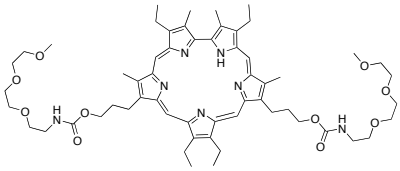
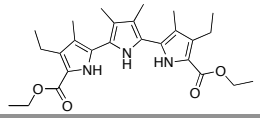
	Carrier	Distribution Coefficient	Cl^- Release at 300 s	Association Constant (K_a), M^{-1}
4.5		0.1585	20%	N/a
4.6		0.0023	82%[a]	5.86 x 10^5
4.7		0.0279	92%	5.80 x 10^5
4.8		N/a	85%	3.02 x 10^5
4.9		N/a	5%	4.80 x 10^5
4.10		0.3999	0%	ca. 100[b], 18,000,000[c]
4.11		N/a	40%	N/a
4.12		N/a	< 5%	< 10^3 [d]

Table 4.1 Chloride binding, extraction, and transport correlation summary. ([a]) Prodigiosin was tested at ¼ the concentration of the other carriers. All of the binding data were determined in acetonitrile using Isothermal calorimetry (ITC), with the exception of the sapphyrin **4.10**, which wasstudied in both methanol ([b]) and dichloromethane([c]) using fluorescence titrations. The chloride binding of **4.12** was too low to be determined accurately by ITC. Due to aggregation, a binding constant for **4.11** could not be determined. Aliquots of tetrabutylammonium chloride in acetonitrile were titrated into a solution of host molecule in acetonitrile at 298K. Mr. Won-Seob Cho is kindly thanked for providing these data. Distribution coefficients were determined by the slope of the data trendline.

4.8 CONCLUSIONS

In both the extraction experiments and the transport experiments, those species which could protonate generally outperformed those species which remained neutral. The relative slopes obtained from the transport data for Carriers **4.11** and **4.12**, both macrocyclic systems, leads the author to conclude that the neutral calixpyrrole could perhaps effect a higher degree of chloride transport if tested over a longer period of time; this, however, remains to be confirmed experimentally. Based on the single datum point provided by the water-soluble sapphyrin with the complications it provides, it is tentatively concluded that size plays a greater role in membrane transport than in extraction, which is an intuitively appealing suggestion. Larger molecules are expected to encounter more difficulties negotiating the packed structure of lipid bilayers.

Anion binding strength follows the same trend as chloride transport, at least among the carriers that could readily protonate. Chloride binding strength alone, is not believed by the author to be solely responsible for transport ability. In fact, one might argue that higher binding constants, at least in polar solvents or aqueous solution, would actually decrease transport rates as the carrier might tend to remain associated with the

anion in one leaflet of the membrane, rather than releasing the anion on the opposite surface. Ideally the carrier would bind most strongly to the anion in the interior of the membrane (in imitation of the transition state of an enzyme-catalyzed reaction), with significantly weaker binding in polar or aqueous media so that the anion is released and the carrier essentially allowed to "turn-over" via back diffusion through the membrane. Such hypotheses were put forward by Lehn and others in the early days of supramolecular chemistry (for cations mostly) in the late 1970s and 1980s.

In summary, this work has shown that despite there being recognized energetic barriers to anion mobilization into organic media from aqueous phases, both extraction and through-membrane transport may be achieved using suitable carriers. Liquid-liquid extraction and membrane transport do not show a 1:1 behavioral correlation. However, liquid-liquid extraction did serve as a useful tool for screening molecules as potential membrane transport agents. The synthetic analogues of prodigiosin transport chloride via an H^+/Cl^- symport mechanism. This work has further shown that the dipyrromethene scaffold is a key unit that "encodes for" efficient chloride transport. A new generation of molecules based on this subunit and possessing stronger hydrogen-bonding moieties could lead to the construction of synthetic systems that display transport efficiencies greater than prodigiosin, whose mechanism of action has been postulated to involve H^+/Cl^- symport. To the extent this proves true, it could provide the basis for improved drug design.

4.9 EXPERIMENTAL

General Information

Fluorescence measurements were carried out on a SPEX-332 spectrofluorimeter. Light absorption and transmittance measurements were carried out on either a Hewlett-Packard diode array spectrophotometer or a Beckman DU 640 spectrophotometer. All solvents were dried prior to use except chloroform. Certified ACS grade chloroform was purchased and used without purification or drying. Deionized water was used for all experiments. Dry Sephadex G-50 packing beads were suspended in a beaker of water and heated in an oven for several hours to fully hydrate and expand prior to use.

Liposome preparation

Materials

Egg L-α-phosphatidylcholine (PTC) and soy phosphatidylserine (PTS) were purchased in chloroform solution from Avanti Polar Lipids, Inc. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) were purchased from both Avanti Polar Lipids Inc. and Northern Lipids Inc. Cholesterol was purchased from Northern Lipids Inc. Chemicals purchased from Northern Lipids Inc. arrived as a white powder and were dissolved in chloroform for storage and use. N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) was purchased from Molecular Probes Inc. and used without further purification. DMSO was dried over molecular sieves. All other chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise specified.

Equipment

A standard rotary evaporator and high vacuum pump were used to effect initial removal of solvent and to form a lipid film. An Avanti Mini-Extruder was used to create liposomes of uniform diameter (0.1, 0.2, 0.4 and 0.8 μm). Membrane filters of 0.2 μm pore size were purchased from Avestin Inc. Membrane filters of 0.1, 0.4 and 0.8 μm pore size were purchased from Avanti Polar Lipids Inc. Spectra/Por Biotech dialysis membranes Cellulose Ester (CE) with a MW 1000 cutoff were purchased from Fisher Scientific. Chloride measurements were taken using an Accumet solid-state, glass body combination ion-selective electrode (ISE) from Fisher Scientific in conjunction with a Jenco model 6209 multimeter purchased from Lazar Research Inc.

Influx Experiments

Preparation of MQAE or $AgNO_3$-containing Liposomes

Lipids were mixed in an 8:2 molar ratio of PTC : PTS as above. After drying, the lipid film was hydrated with a 2-5 mM aqueous indicator solution or an aqueous solution of silver nitrate. The crude suspension was subjected to 9 freeze-thaw cycles by repeatedly immersing the flask in liquid nitrogen and then in a warm water bath. The suspension was left to sit for 1 hour before extrusion through a polycarbonate membrane with 200 nm pores (or 100, 400, 800 nm) using an Avanti Mini Extruder. The suspension was extruded for a total of 39 passes through the membrane filter to create small, unilamellar vesicles (SUVs). To remove extra-liposomal indicator or silver nitrate, the SUV suspension was passed through a Sephadex G-50 size-exclusion column and diluted up to a 1 mM lipid concentration.

Fluorescent Detection

To the liposome suspension, an experimental carrier was added (2-5 mM in methanol or DMSO). A salt gradient was established by the addition of a NaCl solution. Fluorescence quenching was monitored over a period of ten minutes using a steady-state fluorimeter and exciting at λ= 355 nm. Liposomes were lysed after ten minutes using Triton-X to provide an endpoint measurement as discussed in the text proper.

Light Scattering Detection

To the liposome suspension, an experimental carrier was added (2-5 mM in methanol or DMSO). A salt gradient was established by the addition of a NaCl solution. A decrease in light transmittance was monitored using a Hewlett-Packard photodiode array spectrophotometer over a period of ten minutes. Liposomes were lysed after ten minutes using Triton-X to provide an endpoint measurement as discussed in the text proper.

Efflux Experiments using Chloride-Selective Electrode

Preparation of liposomes for efflux experiments

1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-Palmitoyl-2-oleoyl-phosphatidylserine (POPS) or egg L-α-phosphatidylcholine (PTC) and soy phosphatidylserine (PTS) (8:2 molar ratio) were mixed in a round bottom flask. The solvent was removed by rotary evaporation to yield a lipid film which was dried under vacuum overnight. The lipid film was hydrated with 500 mM NaCl and vortexed to yield a crude 30 mM (by lipid concentration) large, multilamellar vesicle (LMV) suspension.

The crude suspension was subjected to 9 freeze-thaw cycles by repeatedly immersing the flask in liquid nitrogen and then in a warm water bath. The suspension was left to sit for 1 hour before extrusion through a polycarbonate membrane with 200 nm pores (or 100, 400, 800 nm) using an Avanti Mini Extruder. The suspension was extruded for a total of 39 passes through the membrane filter to create small, unilamellar vesicles (SUVs). This SUV suspension was sealed in dialysis tubing with nylon clamps and dialyzed overnight against 500 mM NaNO3 in a 2L beaker to remove extra-liposomal NaCl. As the component lipids are light-sensitive, the containers were covered with aluminum foil at all times.

Efflux experimental procedure

The electrode was calibrated using 100 mL standard solutions of NaCl ranging from 1-10,000 ppm, to which 2 mL of a 5 M $NaNO_3$ had been added as an ionic strength adjuster (ISA). Readings (in mV) were plotted against a log scale of the solution concentrations. From this standard curve, experimental concentrations were determined. Concentrated liposome suspensions were diluted to 1 mM (in total lipid concentration) suspensions with a buffer/salt solution consisting of 500 mM $NaNO_3$, 5 mM TES (pH 7.4) or 5 mM MES (pH 5.5). The electrode was inserted into the suspension and allowed to stabilize before addition of 1-5 μL of 5 mM carrier solutions in DMSO. The mV readings were recorded every 10 seconds for the first minute and every 30 seconds for four minutes more. After 5 minutes, the liposomes were lysed by the addition of detergent (100 mM Triton-X or 100 mM octaethylene glycol monododecyl ether) and a final mV reading was recorded. The final reading was taken to be 100% Cl^- release as discussed in the text proper.

Fluorescence experiments using HPTS

Liposomes were prepared in as previously described, with the caveat that the lipid film was hydrated with an aqueous solution containing 10 mM HPTS and 500 mM NaCl. Following extrusion, the liposomes were purified on a Sephadex G-25 gel filtration column to remove the extra-vesicular dye. The liposome suspension was diluted to a final lipid concentration of 1 mM. 100 μL of liposome suspension were added to 2.0 mL buffer. The excitation spectrum was measured with emission at 511 nm prior to addition of each carrier solution. 2.0 μL of each carrier solution (5 mM carrier in DMSO) were added via syringe and the excitation scans were repeated every 1 minute for 10 minutes. The liposomes were then lysed by the addition of 20 μL 100 mM octaethylene glycol monododecyl ether and a final excitation scan was taken.

Leakage test protocol

Preparation of carboxyfluorescein-loaded liposomes

1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) or egg L-α-phosphatidylcholine (PTC) and soy phosphatidylserine (PTS) (8:2 molar ratio) were mixed in a round bottom flask. The solvent was removed by rotary evaporation to yield a lipid film, which was dried under vacuum overnight. The lipid film was hydrated with the addition of a 0.5 mM solution of carboxyfluorescein in buffer solution (of 500 mM $NaNO_3$, 5 mM TES at pH 7.4) and vortexed to yield a crude 30 mM (by lipid concentration) large, multilamellar vesicle (LMV) suspension. The crude suspension was subjected to 9 freeze-thaw cycles by repeatedly immersing the flask in liquid nitrogen and warm water baths. The suspension was left to sit for 1 hour before extrusion through a polycarbonate membrane with 200

nm pores using an Avanti Mini Extruder. The suspension was extruded for a total of 39 passes through the membrane filter to create small, unilamellar vesicles (SUVs). Unencapsulated dye was removed from the liposome suspension by subjecting the crude material to size-exclusion chromatography (15 cm x 1 cm packed material), eluting with buffer solution. Prior to chromatography, Sephadex G-25 was suspended in a 600 mL beaker of water and placed in an oven at 60°C in order to fully expand the beads. The liposome suspension was collected and diluted with more buffer to give a final lipid concentration of 1 mM.

Leakage protocol

A 2.5 mL aliquot of a dye-loaded liposome suspension was added to a fluorescence cuvette. An initial T_0 fluorescence reading was taken (λ_{ex} = 430 nm, λ_{em} = 512). The initial reading was taken to be basal leakage. The carrier solution, 2.0 μL of a 1.0 mM DMSO solution, was added and the fluorescence monitored over time. Readings were taken every two minutes for eight minutes. The liposomes were lysed by the addition of 10 μL octaethylene glycol monododecyl ether and a final fluorescence measurement was taken. The final measurement was taken to be 100% leakage.

Carrier partitioning experiments carried out at The University of Texas at Austin

Solutions of each carrier (10 mM) were prepared in chloroform. A buffer solution consisting of 500 mM $NaNO_3$ and 5 mM TES, adjusted to pH 7.4 using concentrated $NaHCO_3$ was used as the aqueous phase. To six 2-mL centrifuge tubes were added carrier solutions in chloroform in the following amounts: 450 μL, 225 μL, 100 μL, 50 μL, 25 μL and 10 μL. An additional volume of chloroform was added to each tube in the following

amounts: 0 μL, 225 μL, 350 μL, 400 μL, 425 μL, and 440 μL, respectively. Each centrifuge tube contained 450 uL of carrier solution in chloroform, in the following concentrations: 10 mM, 5 mM, 2.2 mM, 1.1 mM, 0.6 mM, 0.2 mM, respectively. Aqueous buffer (450 μL) was added to each centrifuge tube. The centrifuge tubes were sealed, examined for leaks, and placed into 50 mL conical vials packed with Kimwipes. The conical vials were taped together, placed on a mechanical rotator in the Anslyn group laboratories, and allowed to equilibrate with rotation for one hour. The vials were then removed and the inner tubes were centrifuged for 10 minutes to re-separate the layers. The layers were then separated and subsampled. Aliquots (10 μL each) of the organic layer were subsampled and diluted to 1 mL with chloroform. Samples of the diluted organic layer (10 μL each) were added to 3.0 mL chloroform in a cuvette. Likewise, 250 μL portions of the aqueous layer were subsampled and added to 3.0 mL samples of the buffer contained in a cuvette. No further dilutions were effected. Absorbances were measured for each phase at each concentration of carrier. From the absorbance data, distribution ratios were calculated using the software program Kaleidagraph.

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Vita

Leah Renee Eller was born in West Chester, Pennsylvania on June 14, 1976, the daughter of Dorothy Marie Eller and Harry George Eller. After completing her secondary education at Solanco High School, she entered Randolph-Macon College in Ashland, Virginia. She conducted original research at R-MC and the University of Georgia on two separate Summer Undergraduate Research Fellowships sponsored by the National Science Foundation. She received at Bachelor of Science from Randolph-Macon College, with honors, in May 1998. In the August 1998 she entered the Graduate School of the University of Texas at Austin. In 1999 she received an Integrative Graduate Education Research Training (IGERT) Fellowship from the University of Texas and the National Science Foundation. In 2001 she won a Henze Teaching Award for excellence in teaching. She has accepted a post-doctoral assignment at Oak Ridge National Laboratory working for Dr. Bruce A. Moyer, leader of the Chemical Separations Group in the Chemical Sciences Division.

Permanent address: 2207 Lawnmont Avenue, Apt. 102, Austin, Texas 78756

This dissertation was typed by the author.